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Original Article

Indapamide suppresses amyloid-β production in cellular models of Alzheimer's disease through regulating BACE1 activity

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Abstract: Amyloid-β (Aβ) peptide is present in high levels in the brains of Alzheimer's disease (AD) patients and is closely associated with the pathogenesis of the disease. In the present study, we investigated the effect of indapamide on Aβ-induced neurotoxicity and Aβ production in cellular models of Alzheimer's disease. We found treatment with indapamide significantly increased the cell viability after Aβ challenge. In addition, we examined the expression level and half-life of amyloid precursor protein (APP) and we found treatment with indapamide extended the half-life of APP. Furthermore, we found the production of Aβ was inhibited after indapamide treatment. Most importantly, in indapamide-treated cells, the expression level of secreted APP-β (sAPPβ) and carboxy-terminal fragment-β (CTFβ) were significantly decreased as well as the inhibited enzymatic activity of β-site APP cleaving enzyme1 (BACE1). Moreover, we also found indapamide could not only suppress the production of Aβ, but also improved the clearance of Aβ. In conclusion, the results suggested that indapamide may inhibit the development of Alzheimer's disease and might be a drug candidate for the treatment of Alzheimer's disease.

Keywords: Indapamide, Alzheimer's disease, amyloid-β, BACE1

Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder currently affecting 28 million people worldwide [1]. In recent years, AD has become the most common form of dementia of the aged population, and the worldwide epidemic of this disease has severely threatened the elderly and brought economic burdens to society [2]. Currently, several hypotheses have been proposed to elucidate AD pathogenesis, and the most well studied and acceptable one is amyloid-β (Aβ) hypothesis. According to this hypothesis, the pathogenesis of AD is associated with the presence of extracellular Aβ aggregates, forming neuritic plaques, as well as intra-neuronal Aβ, probably due to alterations in the mechanisms of generation and/or clearance of amyloid in the brain during aging [3]. Therefore, decreasing Aβ production, inhibiting Aβ aggregation and cytotoxicity, improving Aβ clearance are appealing therapeutics for the treatment of AD.

Aβ is composed of 38-42 amino acid residues and is generated by the sequential cleavages of amyloid precursorprotein (APP) by β- and γ-secretase [4]. The β-site APP cleaving enzyme 1 (BACE1) is responsible for β-secretase activity, it cleavages APP and generate soluble APPβ (sAPPβ) and membrane-associated APP carboxyl terminal fragment-β (CTFβ). Aβ is subsequently released from CTFβ by cleavage of γ-secretase [5]. Alternatively, APP could be cleaved by α-secretase at the α-site to generate soluble APPα (sAPPα) which has been shown to exert a neuroprotective effect and prevent the formation of Aβ [6]. Previous studies revealed that the increased expression of BACE1 is correlated with the progression of AD [7, 8], and variety of BACE1 inhibitors including antibodies, peptides, and synthetic and natural compounds were reported to have potential application in AD treatment [9]. Recently, various BACE1 inhibitors have been reported to be suitable for clinical trials [10, 11], which means that the extending researches of the BACE1 inhibitors have great significance for the treatment of AD.

Indapamide is a thiazide-like diuretic that widely used in the therapy of hypertension, as well

as decompensated heart failure. In the present study, we were aiming to investigate the effect of indapamide on AD. We found in the cell models of AD, treatment with indapamide significantly increased the cell viability, extended the half-life of APP, inhibited the production and improved the clearance of Aβ. Interestingly, we found in indapamide-treated cells, the expression level of secreted APP-β (sAPPβ) and carboxy-terminal fragment-β (CTFβ) was significantly decreased as well as the inhibited enzymatic activity of β-site APP cleaving enzyme 1 (BACE1). Overall, in this study, we suggested the protective effect of indapamide on AD and suggested indapamide as a potential drug for treatment of AD.

Material and methods

Materials and cell culture

Indapamide was provided by Laboratory of pharmaceutical chemistry, Shenyang Pharmaceutical University. Human $A\beta_{40}$ and $A\beta_{42}$ were obtained from Sigma-Aldrich. SH-SY5Y cells and HEK293 cells expressing APP Swedish mutant$^{K595N/M596L}$ (HEK293-APPsw) cells were cultured in DMEM medium (4,500 mg/L of D-glucose with 10% FBS) in a cell culture incubator at 37°C with 95% air and 5% CO_2.

Cell viability and apoptosis measurement

Cell viability was assessed using conventional MTT reduction assay as described [12]. SH-SY5Y cells and HEK293-APPsw cells were plated in 96-well plates. The cells were pre-incubated with indicated dose of indapamide for 4 h and exposed to 10 μM Aβ for 24 h. 20 μl of MTT stock solution (5 mg/ml) was added to the culture medium for treating another 4 h at 37°C, followed by the administration of DMSO and detection of the absorbance at 570 nm with a Synergy HT Multi-Mode Microplate Reader (Bio-Teck, USA). Cell apoptosis was measured by Hoechst staining [13]. After pre-incubated with or without indapamide, the cells on coverslips were fixed in 4% paraformaldehyde for 20 min, and then stained with Hoechst 33258 for 15 min. Nuclear morphology was viewed using a fluorescence microscope. The number of cells with apoptotic morphology appearing condensed or fragmented nuclei was counted.

Half-life detection

SH-SY5Y cells and HEK293-APPsw cells were pre-incubated with indicated dose of indapamide for 4 h, 100 μg/ml cycloheximide (CHX) was added into the culture medium for indicated times. The cellular APP was detected by immunoblotting followed by the quantification. APP level at time 0 h was defined as 100%.

ELISA assay

ELISA assay was used to detect the intracellular and extracellular levels of Aβ. Cells supernatants were collected 4 hours post-indapamide-treatment for quantification of extracellular Aβ levels. At the same time cells were lysed and intracellular Aβ levels were measured. Human $A\beta_{40}$ and $A\beta_{42}$ levels were measured by ELISA kits following the manufacture's protocol (Invitrogen, #KHB3481 and #KHB3441).

Western blot analysis

Western blot analysis was performed and antibodies were used as previously reported [14]. SH-SY5Y cells and HEK293-APPsw cells were pre-incubated with indicated dose of indapamide for 4 h, and the cells were lysed by RIPA. Samples were electrophoresed on a 5-20% or 10% SDS-polyacrylamide gel and detected with individual antibodies.

BACE1 activity assay

BACE1 activity assay was performed as described [15]. BACE1 activity assay was examined using commercial assay kits from Millipore following the manufacturer's instructions. Fluorescence intensity was measured with amicroplate reader (Spectra Max) at an excitation wavelength of 345 nm and an emission wavelength of 505 nm.

Intracellular Aβ clearance assay

Intracellular Aβ clearance in SH-SY5Y cells and HEK293-APPsw cells was detected as previously reported [16]. Briefly, the cells were pre-incubated with indicated dose of indapamide for 4 h and then cells were treated with soluble $A\beta_{40}$ or for $A\beta_{42}$ for 3 h, followed by lysis in 50 mmol/L Tris buffer containing 1% SDS and a protease inhibitor cocktail. Protein concentration was measured using BCA protein assay

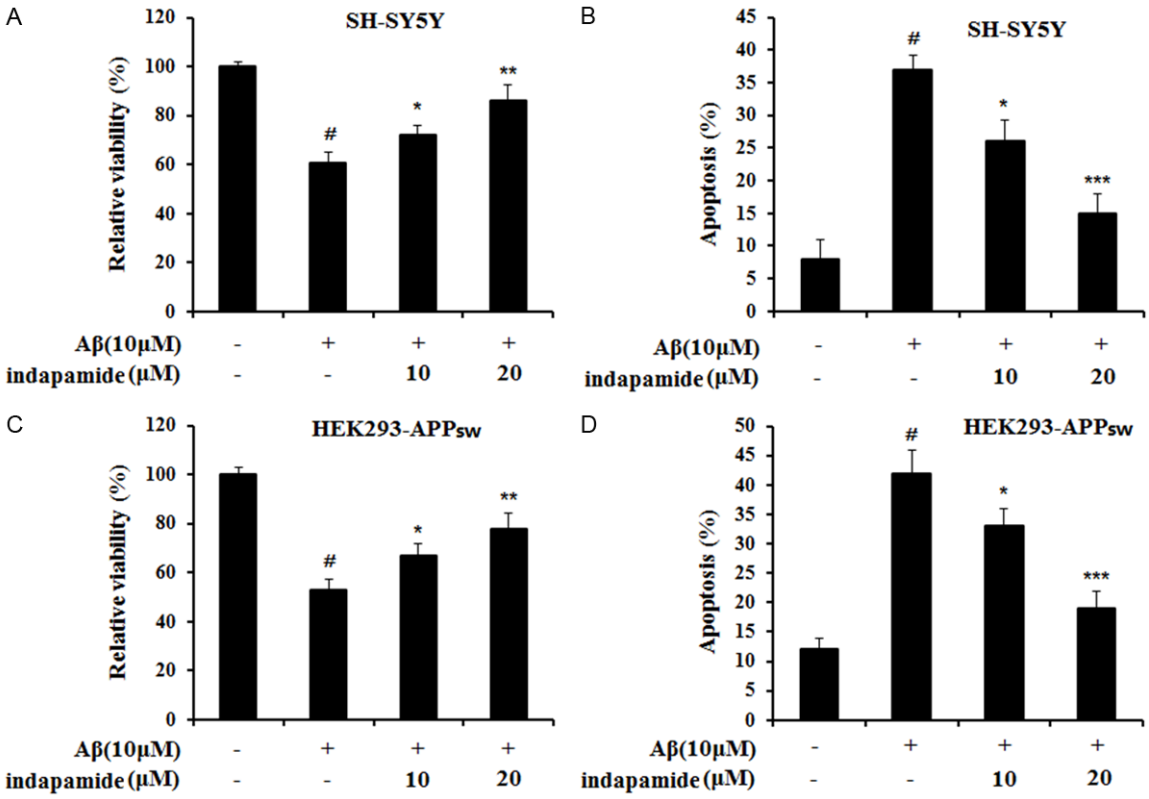

Figure 1. Indapamide protects cells against Aβ-induced cell death and apoptosis. The cells were pre-incubated with or without indicated dose of indapamide for 4 h and exposed to 10 μM Aβ for 24 h. The cell viability and apoptosis was examined. A, B. Cell viability and apoptosis in SH-SY5Y cells. C, D. Cell viability and apoptosis in HEK293-APPsw cells. *P < 0.05, **P < 0.01, ***P < 0.001 versus group treated with only Aβ, #P < 0.05 versus untreated group. Data are representative of three independent experiments (mean ± SD).

kits, and intracellular Aβ peptide was examined using ELISA and normalized to the total protein.

Statistical analysis

All data are presented as mean ± S.D. of three independent experiments. Statistical significance was determined with the two-tailed student's t test to compare two groups. One-way ANOVA was performed to compare three or more groups. If the ANOVA analysis was significant, the Newman-Keuls test was applied for comparison between each two groups. A *p* value of less than 0.05 considered statistically significant.

Results

Indapamide protects cells against Aβ-induced cell death and apoptosis

To investigate the effect of indapamide on AD, we firstly examined the effect of indapamide on cell death and apoptosis induced by Aβ in well studied AD cell models: Human neuroblastoma SH-SY5Y cells and HEK293-APPsw cells that stably expressed Swedish mutant APP [14, 17, 18]. As shown in **Figure 1A**, treatment with indapamide significantly increased the cell viability of SH-SY5Y cells after exposed to Aβ. In addition, the percent of apoptotic cells was decreased after indapamide treatment in dose dependent manner (**Figure 1B**). Consistent with these results, the data in HEK293-APPsw cells also showed that indapamide protected the cells against Aβ-induced cell death and apoptosis (**Figure 1C** and **1D**).

Half-life of APP was extended by the treatment of indapamide

Aβ formation occurs via sequential proteolytic processing of APP and is catalyzed by β- and γ-secretases. We supposed that indapamide affect cell viability via regulation of Aβ production, thus we firstly investigated the effect of

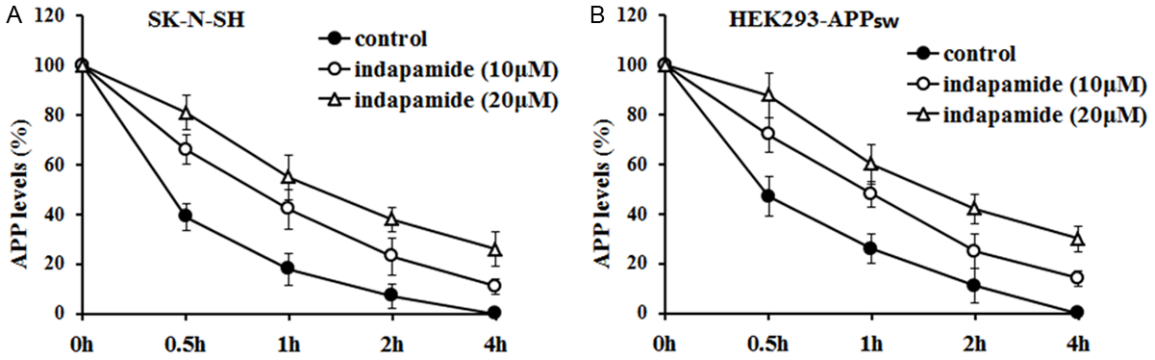

Figure 2. Half-life of APP was extended by the treatment of indapamide. A, B. Half-life of APP was examined in SH-SY5Y cells and HEK293-APPsw cells after indapamide treatment. Data are representative of three independent experiments (mean ± SD).

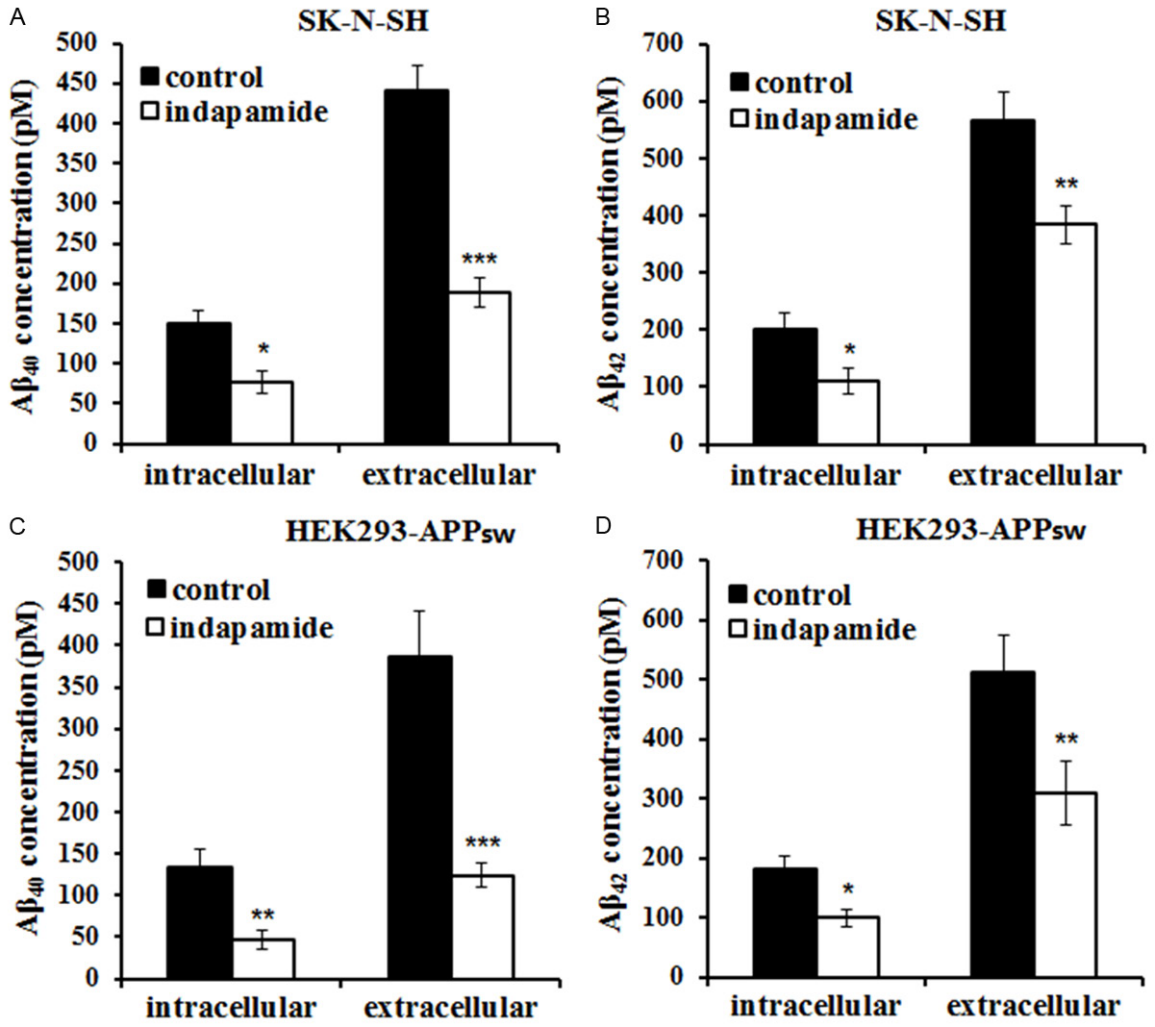

Figure 3. Indapamide decreases intracellular and extracellular levels of Aβ. ELISA assay was performed to examine the intracellular and extracellular of $Aβ_{40}$ or $Aβ_{42}$ in SH-SY5Y cells (A, B), HEK293-APPsw cells (C, D) with or without indapamide treatment. *P < 0.05, **P < 0.01, ***P < 0.001. Data are representative of three independent experiments (mean ± SD).

indapamide on APP degradation. We found that in SH-SY5Y cells, the degradation of APP with a half-life of ~0.4 h, however the degradation of APP was greatly attenuated after indapamide

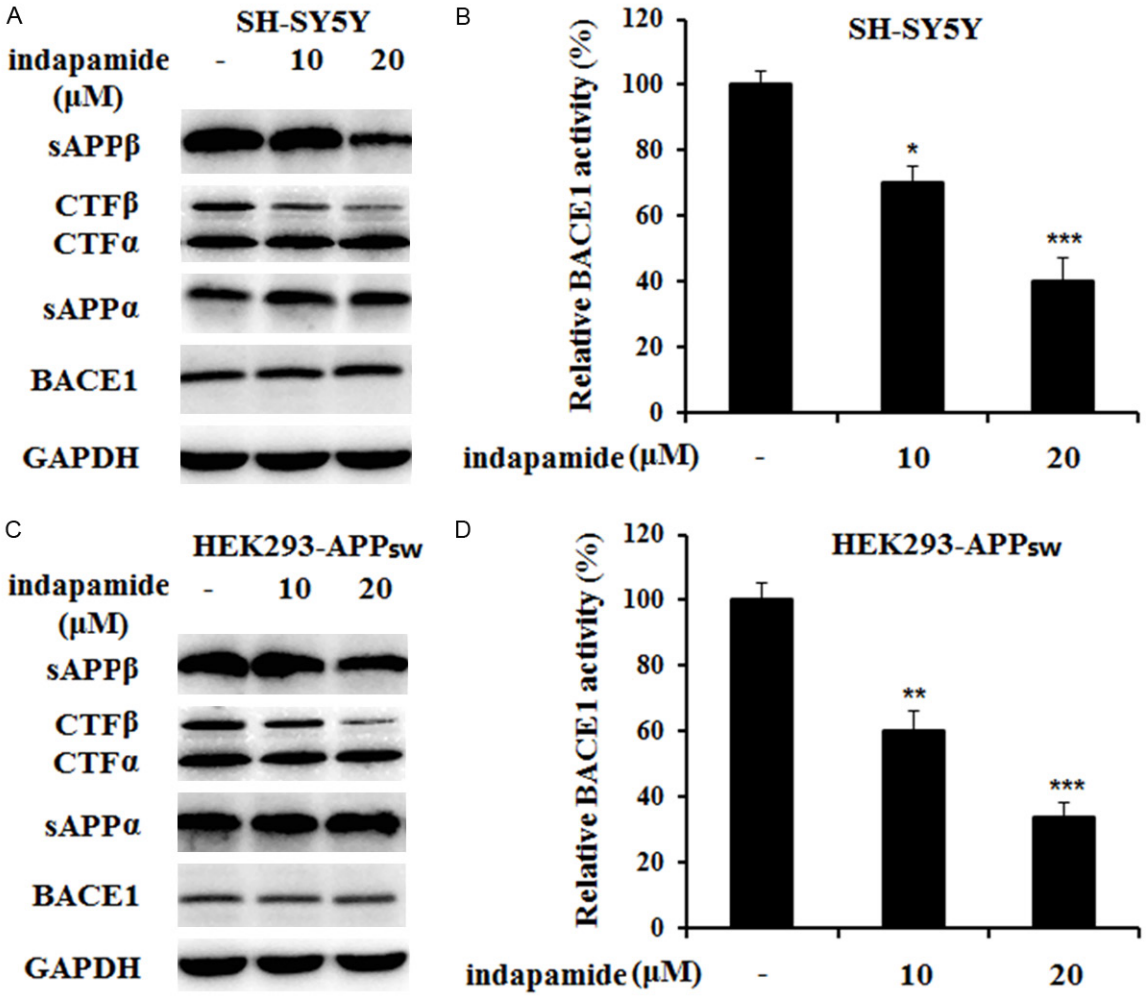

Figure 4. Indapamide suppresses the activity of BACE1. (A, C) The protein levels of sAPPα, sAPPβ, CTFα and CTFβ as well as BACE1 in indapamide treated SH-SY5Y cells (A) and HEK293-APPsw cells (C). (B, D) The activity of BACE1 in indapamide treated SH-SY5Y cells (B) and HEK293-APPsw cells (D). *P < 0.05, **P < 0.01, ***P < 0.001. Data are representative of three independent experiments (mean ± SD).

added (**Figure 2A**). Consistent with these data, we observed in HEK293-APPsw cells, APP was degraded more rapidly in cells without indapamide treatment (**Figure 2B**).

Indapamide decreases intracellular and extracellular levels of Aβ

Accumulation of Aβ is one of the characteristic features found in patients with AD. We used ELISA assay to examine the levels of Aβ in cells and culture medium. As shown in **Figure 3A**, the levels of both intracellular and secreted levels of $A\beta_{40}$ and $A\beta_{42}$ were significantly decreased in cells treated with indapamide. Similar results were observed in HEK293-APPsw cells (**Figure 3B**).

Indapamide suppresses the activity of BACE1

Based on the results we obtained, we supposed that indapamide regulates the production of Aβ via the directly cleavage of APP. Therefore we investigated the protein levels of products in APP cleavage, such as sAPPα, sAPPβ, CTFα and CTFβ as well as BACE1. As shown in **Figure 4A**, we found the protein levels of sAPPα, CTFα and BACE1 were not affected by indapamide challenge, but the levels of sAPPβ and CTFβ were obviously decreased after indapamide treatment in a dose-dependent manner. We also found in SH-SY5Y cells, indapamide treatment significantly inhibited the activity of BACE1 (**Figure 4B**), although the protein level of BACE1 was not changed by

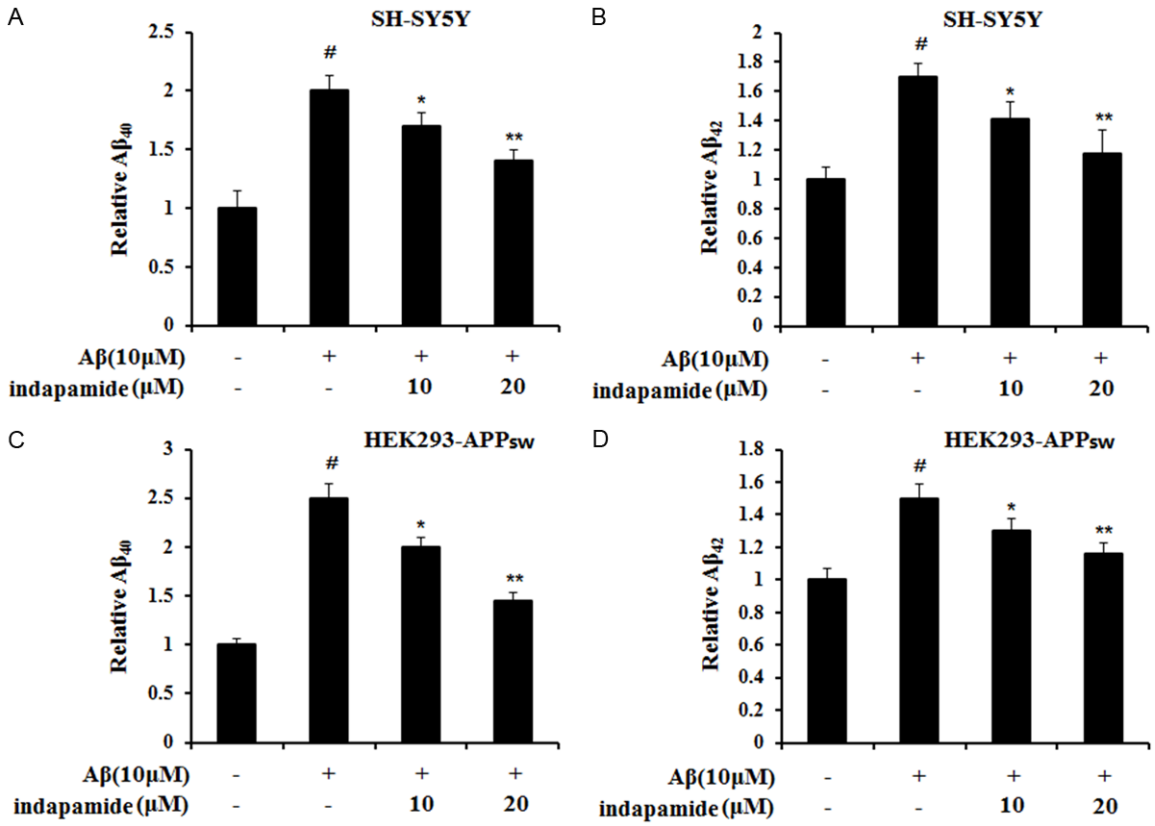

Figure 5. Indapamide promotes the clearance of exogenous Aβ. (A, C) The intracellular $Aβ_{40}$ clearance was examined in indapamide treated SH-SY5Y cells (A) and HEK293-APPsw cells (C). (B, D) The intracellular $Aβ_{42}$ clearance was examined in indapamide treated SH-SY5Y cells (B) and HEK293-APPsw cells (D). *P < 0.05, **P < 0.01, ***P < 0.001 versus group treated with only Aβ, #P < 0.05 versus untreated group. Data are representative of three independent experiments (mean ± SD).

indapamide (**Figure 4A**). Similar results were also observed in HEK293-APPsw cells (**Figure 4C** and **4D**). These results indicated that indapamide suppressed the activity of BACE1, therefore inhibited the production of Aβ.

Indapamide promotes the clearance of exogenous Aβ

Because Aβ levels involve a dynamic equilibrium between Aβ production and clearance, we also investigated the potential effect of indapamide on exogenous Aβ clearance in SH-SY5Y cells and HEK293-APPsw cells. We added exogenous $Aβ_{40}$ or $Aβ_{42}$ into culture medium, 3 hours later we harvested the cells and examined the intracellular levels of $Aβ_{40}$ or $Aβ_{42}$. The data indicated that both in SH-SY5Y cells and HEK293-APPsw cells, indapamide enhanced exogenous $Aβ_{40}$ clearance with a dose-dependent manner (**Figure 5A** and **5C**), as well as the improved clearance of $Aβ_{42}$ (**Figure 5B** and **5D**).

Discussion

In the present study, we demonstrated the function of indapamide in AD cell model. To the best of our knowledge, this study is the first publication showing the potential effect of indapamide on AD.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder clinically characterized by an insidious onset and slow deterioration in cognition and is the most common cause of dementia in the elderly [19]. Recent studies also found that AD is characterized by senile plaques consisting of Aβ peptide, which derived by the proteolytic cleavage of APP [20]. Elucidation of factors that modulate Aβ has been shown to be crucial for AD intervention [21]. Treatment with Liver X receptor β (LXRβ) agonist T0901317 was found potentially reduce brain Aβ generation by inhibiting Aβ production and promoting Aβ degradation [22]. It has also

been reported that small molecule LX2343 significantly ameliorate cognitive dysfunction in APP/PS1 transgenic mice via both Aβ production inhibition and clearance promotion [16]. Similar with their findings, in the present research, we found treatment with indapamide could obviously suppress the production of Aβ and promote the degradation of Aβ, resulted in the decreased Aβ aggregation.

β-site APP-cleaving enzyme 1 (BACE1) is a membrane-bound aspartic protease and the rate-limiting step in Aβ generation, which is responsible for β-secretase cleavage of APP [23]. It is of great significance to effectively regulate the activity of BACE1 and its expression in the treatment of AD, and various studies have been reported in this regard. Recently, endosome-associated deubiquitinating enzyme USP8 was found to regulate BACE1 enzyme ubiquitination and degradationin a lysosome dependent manner [24]. In addition, small molecules such as Biochanin A and aminohydantoins were also identified as potent and selective BACE1 inhibitors [25, 26]. As a supplement, in the present study, we found indapamide could also significantly inhibit BACE1 activity, but not affect the protein expression of BACE1, leading to the decreased production of Aβ in AD cell model.

Thiazides are widely used to treat hypertension. Indapamide has been used for 20 years as an antihypertensive drug and is well tolerated even in the elderly [27]. In the recent days, other functions of indapamide have been revealed by researches. Indapamide was found to increase osteoblast-like cell proliferation and decreased bone resorption [28]. It has also been reported that indapamide has protective effect on ischemia-induced injury and barrier dysfunction in mouse brain microvascular endothelial cells [29]. Several epidemiological studies have shown that high mid-life blood pressure is related to the development of Alzheimer's disease in later life [30, 31], based on these findings, we investigated the effect of indapamide in AD cell model to illustrate the function of indapamide in AD. As we shown in the present research, our results indicated that indapamide treatment could greatly attenuate the cell death and apoptosis induced by Aβ, moreover, indapamide inhibited the Aβ production and promoted the Aβ degradation in AD cell model.

In conclusion, we investigated the function of indapamide in AD cell model and we found the activity of BACE1 was significantly inhibited by indapamide treatment, leading to the decreased Aβ aggregation. Interestingly, we also found treatment of indapamide promoted the clearance and degradation of Aβ, but the related mechanisms and how does indapamide affect BACE1 activity still need further study.

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Disclosure of conflict of interest

None.

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