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Resveratrol-loaded polymeric micelles protect cells from Aβ-induced oxidative stress

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ABSTRACT

Resveratrol has been reported to protect several types of cells against β-amyloid peptide (Aβ) toxicity by scavenging reactive oxygen species (ROS) and inactivating caspase-3. However, other studies found that long-term treatment with resveratrol inhibited cells by inducing ROS generation and activating caspase-3. In the current report, a 48-h incubation of resveratrol at the concentrations of 5 and 10 μM significantly attenuated the viability of PC12 cells and a 12-h pre-incubation of resveratrol did not protect PC12 cells against Aβ exposure (even further inhibited PC12 cells at the concentrations of 10 μM) by acting as a pro-oxidant. Due to the lipophilicity of resveratrol, resveratrol-loaded polymeric micelles basing on amphiphilic block copolymer were developed. Then, the effects of resveratrol-loaded polymeric micelles on the viability and Aβ protection of PC12 cells were investigated. At the equivalent concentrations of 5 and 10 μM resveratrol, a 48-h incubation of resveratrol-loaded nanoparticles did not show toxicity to cells, while 12-h pre-incubation of resveratrol-loaded nanoparticles protected PC12 cells from Aβ-induced damage in a dose dependent manner (1–10 μM) by attenuating intracellular oxidative stress and caspase-3 activity. Further investigations are absolutely needed to evaluate the feasibility and advantages of in vivo applications of resveratrol-loaded nanoparticles.

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly. Though the exact mechanism of AD has not been fully clarified, it is undoubted that β-amyloid peptide (Aβ)-induced oxidative stress plays an important role in disease process. Resveratrol (trans-3,4,5-trihydroxystilbene, Res), a natural polyphenol, mainly found in grapes and red wine, has been reported as a powerful antioxidant for the therapy of AD (Jang and Surh, 2003; Russo et al., 2003; Sharma and Gupta, 2002). In vitro studies, which were executed in PC12 cells, human umbilical vein endothelial cells (HUVECs), SHSY5Y neuroblastoma cells and hippocampal neuronal cell cultures, had found that resveratrol protected cells from oxidative stress and apoptosis induced by Aβ (Jang and Surh, 2003; Russo et al., 2003; Sharma and Gupta, 2002; Savaskan et al., 2003; Han et al., 2004). In these studies, it was true that resveratrol exhibited neuroprotective effect at the concentration range of 5–25 μM when used as a several-hour pretreatment. However, there are still two aspects of this potential drug to be further explored.

First, several recent lines of evidences from in vitro and in vivo studies suggested that resveratrol demonstrated cytotoxic effect in a dose- and time-dependent manner (Athar et al., 2007; Juan et al., 2008). Resveratrol could hinder tumor growth, wound healing, endothelial cell growth and angiogenesis in healthy tissues in a dose dependent manner (Bråkenhielm et al., 2001). A several-month study on rats founded that resveratrol potentiated a death signal by down-regulating redox proteins and up-regulating pro-apoptotic proteins when its concentration increased to 1.1–5.7 μM in plasma (Jocelyn et al., 2008). It is difficult to estimate the concentrations in tissues, which has been shown to be in the nanomolar range and much lower than those used in vitro. Furthermore, results from in vitro studies cannot be directly compared with those from

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in vivo due to the complex pharmacokinetics and pharmacodynamics of in vivo administration. Nevertheless, both in vitro and in vivo studies indicate the possible toxicity of resveratrol. AD is a chronic progressive disorder and the plausible treating/preventing drugs (such as resveratrol) should be started early and maintained over a long span of time, so it is necessary to develop a long-term treatment/prevention without long-term toxicity. Up to date, it is still unknown about the long-term effect of resveratrol on AD model at those cell culture-based concentrations.

Second, the importance of antioxidant properties of trans-resveratrol in AD was highlighted by cell culture studies (Anekonda, 2006). Despite the unquestionable protective activity in several in vitro models, clinical studies demonstrated only minimal effect in humans and this was related to the pharmacokinetics of these substances (Mancuso et al., 2007). In reality, trans-resveratrol in plasma is very sparse (only several micromoles after addition) with short half-life time because of its poor water-solubility (<1 μM) and unstability (Athar et al., 2007; Juan et al., 2008; Jocelyn et al., 2008). The antioxidant role of resveratrol seems to be considerably diluted in vivo during the extensive and rapid metabolization to glucuronic and sulfate metabolites of resveratrol (Anekonda, 2006).

One of recent progresses in drug delivery has focused on improving drug delivery for neurodegenerative disease using nanomedicine and polymer techniques (Kabanov and Gendelman, 2007). New drug delivery systems may be desirable and useful for the therapeutic use of antioxidants in human neurodegenerative diseases (Mancuso et al., 2007). Among them, amphiphilic block copolymer-based polymeric micelles receive most attention because they can self-assemble into nanoparticles with hydrophilic outer shells and hydrophobic inner cores, which capture hydrophobic drug in the cores and disperse easily in solution with the protection of hydrophilic shells. These drug-loaded polymeric micelles are not bigger than 100 nm, which are easy to be internalized by cells (Kabanov and Gendelman, 2007). By incorporating in these nanoparticles, drugs are prevented from being quickly degraded (Hu et al., 2003; Zhang et al., 2004) and enabled to sustained release at the expected site (Manconi et al., 2007). The hydrophobicity of resveratrol makes it possible that Res acts as a model drug to develop Res-loaded nanoparticles. It also brings about the assumption that water-soluble, biocompatible, injectable, and controlled release Res-loaded nanoparticles can be used as a potential therapeutic tool for Aβ-related oxidative stress. Undoubtedly, it is necessary to assess the cytotoxicity and the protection against Aβ-related toxicity of Res-loaded nanoparticles in vitro before in vivo application.

In the present study, Res-loaded nanoparticles were developed and characterized. Furthermore, by analysis of cell viability assay, intracellular ROS level and caspase-3 activity, the long-term effects of Res-loaded nanoparticles (at the concentration range of 0–10 μM) on cultured PC12 cell model were assessed and compared with those of free resveratrol in absence or presence of Aβ.

2. Materials and methods

2.1. Materials

Resveratrol, β-amyloid peptide ($A\beta_{1-42}$), methoxy-polyethyleneglycol (mPEG, MW: 4 and 10 kDa), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,7-dichlorofluorescein diacetate (H_2DCF-DA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). ε-Caprolactone (ε-CL, Aldrich) was purified by drying over CaH_2 at room temperature and distilling under reduced pressure. Stannous octoate (Sigma) was used as-received. All other chemicals of analytical grade were used without further purification. PC12 cell line was obtained from Shanghai Institute of Cell Biology (Shanghai, China). $A\beta_{1-42}$ stock solution (1 mM) was prepared in deionized water and stored at −20 °C. The $A\beta_{1-42}$ stock solution was diluted to desired concentration immediately before use. Resveratrol was dissolved in DMSO to the concentration of 50 mM and further diluted with cold phosphate buffered saline (PBS). The final concentration of DMSO in culture media was 0.1%.

2.2. Preparation of nanoparticles

In the present report, we chose poly-caprolactone (PCL) as the hydrophobic core of the block copolymer for its good drug encapsulation ability and polyethyleneglycol (PEG) as the hydrophilic shell because of its hydrophilicity, biocompatibility, low toxicity, negligible antigenicity and immunogenicity (Herold et al., 1989; Richter and Akerblom, 1983) to synthesize Res-loaded nanoparticles. According to previous work, the drug incorporation exerts no change on particle size, while the chemical composition of the nanoparticles mainly influences the particle size. The mean diameter of PCL20k–PEG4k is around 70 nm and drug-loaded nanoparticles formed by PCL20k–PEG4k have higher drug loading content and encapsulation efficiency (Li et al., 2008). Therefore, we applied PEG4k–PCL20k nanoparticles in the following evaluation. Res-loaded nanoparticles were prepared by nano-precipitation method with minor modification (Hu et al., 2007). To explain briefly, 10 mg mPEG–PCL block copolymers and a predetermined amount of Res were dissolved in an aliquot of acetone. The obtained organic solution was added dropwise into 10 times volumes of distilled water under moderate stirring at 25 °C. The solution was put into a dialysis bag (MWCO 12000) and then was immerged in the redistilled water to remove acetone thoroughly. The obtained bluish aqueous solution was filtered through a 0.22 μm filter membrane to remove non-incorporated drugs and copolymer aggregates. Blank nanoparticles were prepared in a similar manner by omitting drug. To visualize the uptake of polymeric micelles by the cells under fluorescence microscopy, green fluorescent coumarin-6 was incorporated into the particles. Coumarin-6 loaded nanoparticles were prepared by replacing Res with coumarin-6. Solutions of Res-loaded nanoparticles and blank nanoparticles were lyophilized by freeze dryer with the presence of cryoprotectant (F68, for medical use, BASF, Germany) for further characterization and utilization. Res-loaded nanoparticles were diluted to desired concentration immediately before use according to the equivalent dose of Res.

2.3. Characterization and in vitro release of Res-loaded nanoparticles

The physical characteristic of Res-loaded nanoparticle (Res-np) was determined by dynamic light scattering (DLS, Brookheaven BI-9000AT, USA), transmission electron microscopy (TEM, JEM-100S, Japan), atomic force microscopy (AFM, SPI3800, Seiko Instruments, Japan) and Fourier transform infrared spectroscopy (FTIR, Perkin-Elmer Paragon 1000 Fourier transform spectrometer).

The drug encapsulation behavior and in vitro release pattern were evaluated by high performance liquid chromatography (HPLC, Shimadzu LC-10AD, Japan) system equipped with a Shimadzu UV detector and an agilent C-18, 5 μm, 200 mm × 4.6 mm RP-HPLC analytical column. The mobile phase consisted of methanol (spectral grade, Merck, Germany)/double-distilled water/glacial acetic acid (48/52/0.05, v/v/v) and was pumped at a flow rate of 1.0 ml/min with determination wavelength of 303 nm. The concentration of Res was determined on the peak area at the retention time of about 4.2 min by reference to a calibration curve. The drug loading con-

tent and the drug entrapment efficiency were presented by Eqs. (1) and (2), respectively:

$$\text{drug loading content (\%)} = \frac{\text{weight of the drug in nanoparticles}}{\text{weight of the nanoparticles}} \times 100 \quad (1)$$

$$\text{encapsulation efficiency (\%)} = \frac{\text{weight of the drug in nanoparticles}}{\text{weight of the feeding drugs}} \times 100 \quad (2)$$

The release of Res from the core–shell polymeric nanoparticles in PBS (pH 7.4) was evaluated by dialysis method. Briefly, 10 mg lyophilized Res-loaded nanoparticles was suspended in 1 ml of 0.1 M PBS (pH 7.4) and then the solution was placed into a pre-swelled dialysis bag with a 12-kDa molecular weight cutoff (Sigma) and immersed into 40 ml 0.1 mol/l PBS (pH 7.4) at 37 °C with gentle agitation. 1 ml samples were withdrawn from the incubation medium and then were measured for Res concentration. After sampling, equal volume of fresh PBS was immediately added into the incubation medium. The concentration of Res released from the nanoparticles was expressed as a percentage of the total Res in the nanoparticles and was plotted as a function of time.

2.4. Nanoparticles uptake by PC12 cells

To assess the efficiency of nanoparticles uptake by PC12 cells, about 5×10^5 PC12 cells were seeded in six-well plates with Dulbcco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum and allowed to adhere at 37 °C with 5% CO_2 for 24 h prior to the assay. The medium was then replaced with fresh DMEM containing coumarin-6 loaded nanoparticles. The final concentration of coumarin-6 in the medium was 4 μg/ml. After 3 h incubation, the monolayers were rinsed three times with PBS to remove excess nanoparticles. The cells were viewed and imaged under a fluorescence microscope according to the green fluorescence of coumarin-6.

2.5. Cell culture and treatment

This procedure was carried out according to previous report with slight modification (Jang et al., 2007). The culture media for PC12 cells was Dulbcco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum. After plated, all the cells adhered at 37 °C with 5% CO_2 for 24 h prior to application. To assess cell viability and ROS level, PC12 cells were plated in 96-well plates at a density of 2×10^4 cells/well. For the detection of caspase-3 activity, cells were plated at a density of 10^6 cells/culture dish (Ø 60 mm). When the long-term effect of Res and Res-loaded nanoparticles was evaluated, PC12 cells were incubated in culture medium containing desired doses of free Res or Res-loaded nanoparticles (the equivalent concentrations of Res were 2, 5, and 10 μM) for 48 h. For the effects of overnight (12 h) pretreatment of Res/Res-np on Aβ-treated PC12 cells, $A\beta_{1-42}$ solution was added to culture medium (final $A\beta_{1-42}$ concentration in culture medium is 10 μM) after pretreatment procedure (the equivalent concentrations of Res were 1, 5, and 10 μM). Then cells were incubated in culture media containing 10 μM $A\beta_{1-42}$ for 24 h. The protective effect of a 2-h pre-incubation of Res on Aβ-treated PC12 cells was also assessed by the assay of cell viability. Controls treated without drugs nor $A\beta_{1-42}$ solution were cultured in medium containing empty PCL–PEG nanoparticles (5 mg/ml), which is non-toxic to cells in our preliminary study (data not shown).

2.6. Determination of cell viability

After incubation, cold PBS (pH 7.4) was utilized to wash the cells carefully and then 50 μl of MTT indicator dye (5 mg/ml in PBS, pH 7.4) was added to each well and incubated for 2 h at 37 °C in the dark. The medium was withdrawn and 200 μl acidified isopropanol (0.33 ml HCl in 100 ml isopropanol) was added to each well and agitated thoroughly to dissolve the formazan crystals. Absorption was measured at 550 nm in Microkinetics reader BT2000 and obtained values were expressed as a percentage of the controls.

2.7. Measurement of intracellular ROS

The intracellular ROS levels were measured by a fluorescent dye staining method. For this detection, PC12 cells were pretreated with free Res or Res-np at the equivalent Res dose of 10 μM. After 12-h pre-incubation, the testing drugs were washed away, and then the cells were cultured with 10 μM $A\beta_{1-42}$ for 24 h. Cells incubated with blank-np and 10 μM $A\beta_{1-42}$ in sequence were defined as positive control, while cells incubated only with blank-np as negative control. Following the exposure to $A\beta_{1-42}$, cells were incubated with 5 mM H_2DCF-DA for 30 min at 37 °C. Then cells were washed three times with cold PBS and the intensity of fluorescence was quantified using a confocal microscope equipped with an argon laser using an excitation wavelength at 485 nm and an emission wavelength at 535 nm. The obtained values were expressed as a percentage of the controls. Fluorescent images of ROS accumulation in cells were obtained under fluorescence microscope according to green fluorescence of DCF. The higher the fluorescence, the greater the ROS production.

2.8. Measurement of caspase-3 activity

The caspase colorimetric protease assay kit was utilized for the determination of caspase-3 activity (Keygen Biotech, Nanjing, China) (Zhang et al., 2007). The cells were grouped according to the definition in Section 2.7. Cells were washed three times with cold PBS and then resuspended in 50 μl of chilled cell lysis buffer [containing 50 mM HEPES (pH 7.4), 5 mM Chaps and 5 mM diothreitol (DTT)]. After incubating on ice for 10 min, cytosolic extract (the supernatant) was obtained by centrifugation (1 min, 10,000 × *g*). After adjusting the protein concentration by the cell lysis buffer, the sample (50 μl) was added to twofold concentrated reaction buffer [50 μl; containing 40 mM HEPES (pH 7.4), 3 mM Chaps, 10 mM DTT and 4 mM EDTA] and DEVD-pNA (caspase-3), then incubated at 37 °C for 4 h (Zhang et al., 2007). After incubation, every sample was read at 405 nm. The obtained values were expressed as a percentage of the control.

2.9. Statistical analysis

All data were expressed as the mean ± S.D. of three independent experiments. Statistical analysis for the comparison of relative groups was based on Student's *t*-test. Significance was accepted at the 0.05 or 0.01 level of probability.

3. Results

3.1. Characterization and in vitro release of Res-loaded nanoparticles

Both TEM and AFM images showed the spherical shape of the polymeric nanoparticles with a smooth surface (Fig. 1A and B). DLS determined the size of Res-loaded nanoparticles were less than 100 nm. The FTIR confirmed that Res was successfully incorporated into the nanoparticles, since its characteristic peaks at 1518 and

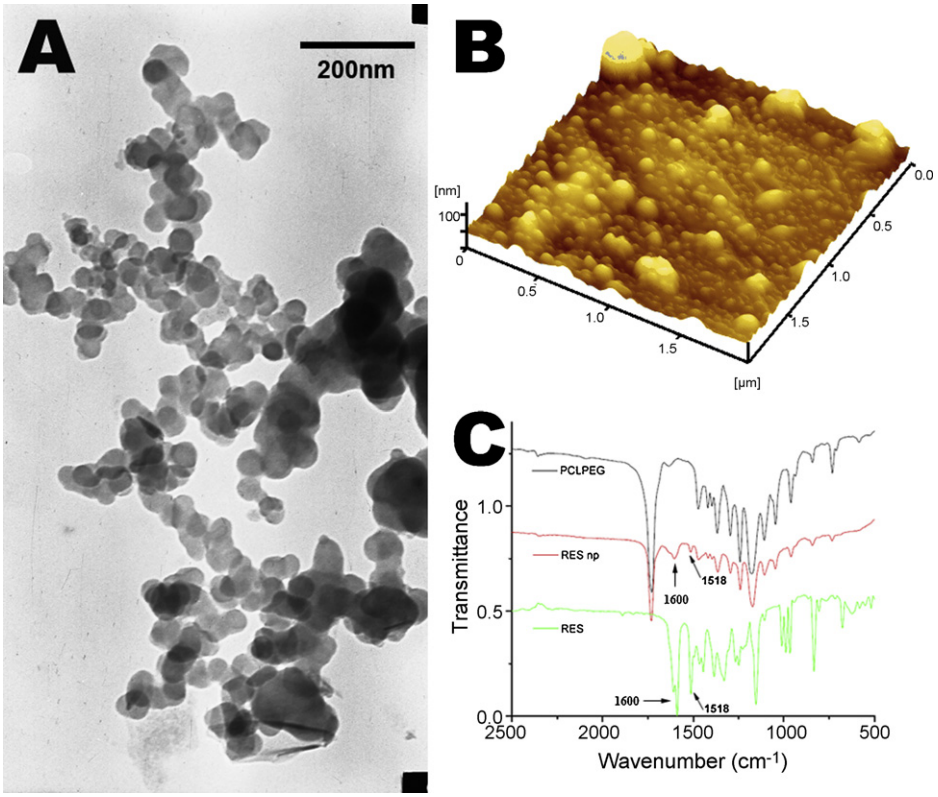

Fig. 1. Physical characteristic images of mPEG–PCL nanoparticles (np). (A) TEM image of mPEG–PCL nanoparticles. (B) AFM image of mPEG–PCL nanoparticles. (C) The FTIR spectrums of free resveratrol (Res), empty nanoparticles (E-np) and resveratrol-loaded nanoparticles (Res-np), the characteristic peaks of resveratrol at 1518 and 1600 cm^{-1} can be differentiated from the background of carriers.

1600 cm^{-1} could be differentiated from the background of carriers (Fig. 1C).

The loading content varies depending on the different feeding ratios. By varying the feeding ratio of copolymer and Res, the highest drug loading content of Res into nanoparticles was about 20%. The highest encapsulation efficiency was about 89%.

3.2. Uptake of Res-loaded nanoparticles by PC12 cells

Fig. 2A and B shows the cellular uptake of nanoparticles by using green fluorescent coumarin-6 as visible marker. 3-h incubation was sufficient for PC12 cells to uptake the nanoparticles. It was obvious that coumarin-6 loaded nanoparticles were localized in the cytoplasm.

Fig. 3 shows the slow release profile of Res-loaded nanoparticles. An initial burst of 35% release in 8 h was found, which could be explained that the affiliation of certain drugs to the surface of the nanoparticles was unavoidable. During the following period, Res released in a sustained manner from the core–shell structure nanoparticles. Finally, 5 days incubation with PBS (pH 7.4) caused an about 60% release.

3.3. Long-term effects of Res and Res-loaded nanoparticles on the viability of PC12 cells in the absence of $A\beta_{1-42}$

Measured by MTT assay, cell viability was significantly inhibited by 48-h incubation of free Res in a dose dependent manner, while 48-h incubation of Res-np did not inhibit PC12 cell viabil-

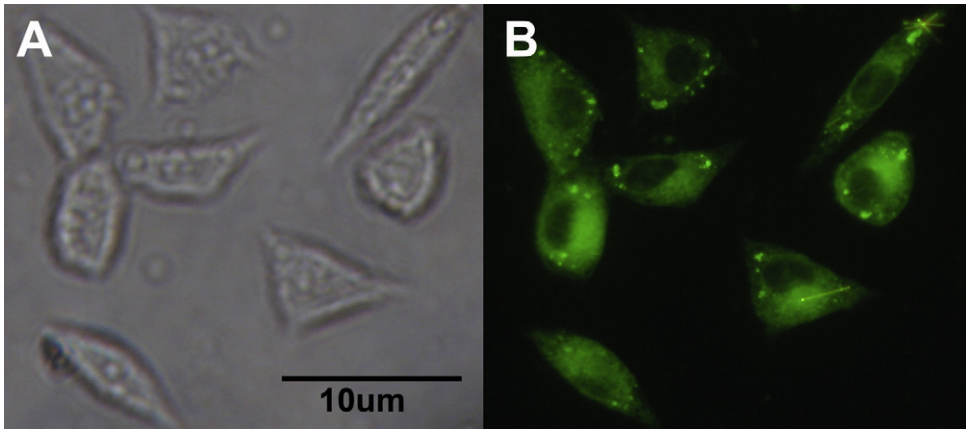

Fig. 2. Uptake of nanoparticles by PC12 cells using green fluorescent coumarin-6 as visible marker. (A and B) Microscopic images of PC12 cells incubated with coumarin-6-loaded nanoparticles for 3 h ((A) bright field; (B) fluorescent field). Obviously, coumarin-6 loaded nanoparticles are well internalized and localized in the cytoplasm.

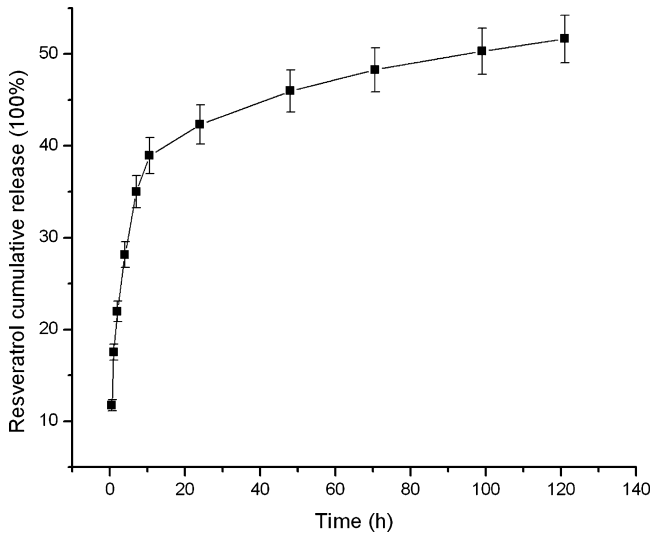

Fig. 3. In vitro release profile of Res-np. The cumulative in vitro release profile of resveratrol from mPEG–PCL nanoparticles. Values represent mean ± S.D. ($n = 3$).

ity. It was noteworthy that 5 μM Res decreased cell viability by 25% ($p < 0.05$) and 10 μM Res made a 36% decrease of cell viability ($p < 0.05$) when compared with control, respectively. In contrast, nanoparticles containing equivalent doses of Res did not do harm to cell viability and nanoparticles containing 5 μM Res even slightly enhanced cell viability. Neither Res nor Res-np was toxic to PC12 cells at the equivalent concentration of 2 μM after 48-h incubation (Fig. 4C).

3.4. Effects of 12-h pretreatment of Res or Res-loaded nanoparticles on the viability of Aβ-treated PC12 cells

Cell inhibition induced by $A\beta_{1-42}$ was in a dose dependent manner and 10 μM $A\beta_{1-42}$ significantly inhibited PC12 cells shown by MTT assay (Fig. 4A). After a 2-h pretreatment, free Res protected cells from Aβ toxicity in a dose dependent manner at the concentration range of 1–10 μM (Fig. 4B). However, 12-h pre-incubation of free Res did not protect cells from $A\beta_{1-42}$ toxicity at the same concentration range. On the contrary, pretreatment with Res-loaded nanoparticles for 12 h could protect cells against $A\beta_{1-42}$ toxicity in a dose dependent manner (Fig. 4D). Noticeably, a 12-h pretreatment with 5 μM or 10 μM Res made less cells survival than those of pretreated with 5 or 10 μM Res-loaded nanoparticles, respectively ($p < 0.05$ for 5 μM, $p < 0.01$ for 10 μM). Furthermore, pretreatment with 10 μM free Res for 12 h demonstrated more cell inhibition ($p < 0.05$) compared to the positive control (10 μM $A\beta_{1-42}$ treated only), which was significantly opposite to the protective effect of 10 μM Res-loaded nanoparticles ($p < 0.05$, compared to the positive control). Accordingly, the single equivalent concentration of Res was set at 10 μM in the following experiments.

3.5. Controlled release of resveratrol reversed intracellular oxidative stress of Aβ-treated PC12 cells

To determine the effects of free Res and Res-np on the oxidative stress of Aβ-treated PC12 cells marked by ROS level, cells were exposed to 10 μM $A\beta_{1-42}$ for 24 h in the presence or absence of pretreatment with testing drugs (resveratrol or Res-np). $A\beta_{1-42}$ treatment made about 100% ($p < 0.05$) increase in ROS level compared to negative control, which was attenuated by pre-incubation

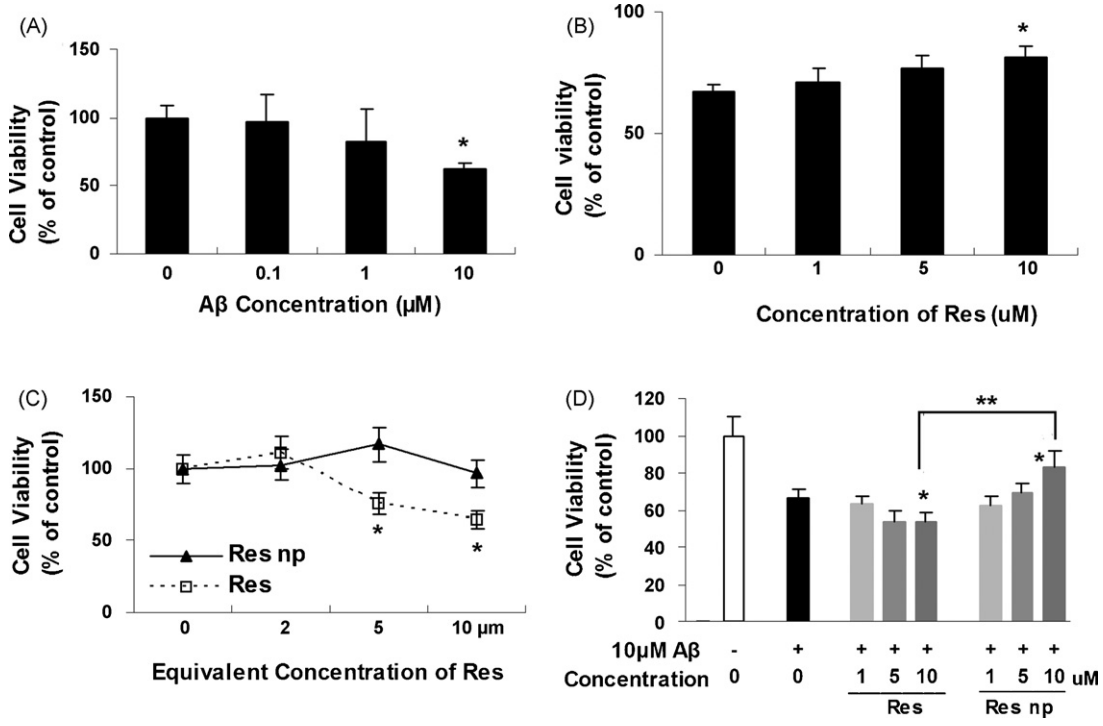

Fig. 4. Viability assay of PC12 cells. Cell viability was assayed by MTT method. (A) Effects of various concentrations of $A\beta_{1-42}$ on PC12 cells. Cell viability was significantly inhibited after exposed to 10 μM $A\beta_{1-42}$ for 48 h. *Significantly different from the control cells ($p < 0.05$). (B) 2-h pre-incubation of free Res protected cells from 10 μM $A\beta_{1-42}$-induced viability inhibition. *Significantly different from the group treated with A_{1-42} alone($p < 0.05$). (C) Effects of various concentrations of Res-np or free Res on cell viability after 48-h incubation. *Significantly different from the control cells ($p < 0.05$). (D) Effects of 12-h pre-incubation of Res or Res-np on 10 μM $A\beta_{1-42}$-induced cell viability inhibition. *Significantly different from the group treated with $A\beta_{1-42}$ alone ($p < 0.05$). **Significant difference between the groups pretreated with 10 μM free Res and 10 μM Res-np ($p < 0.05$). Values represent mean ± S.D. ($n = 3$).

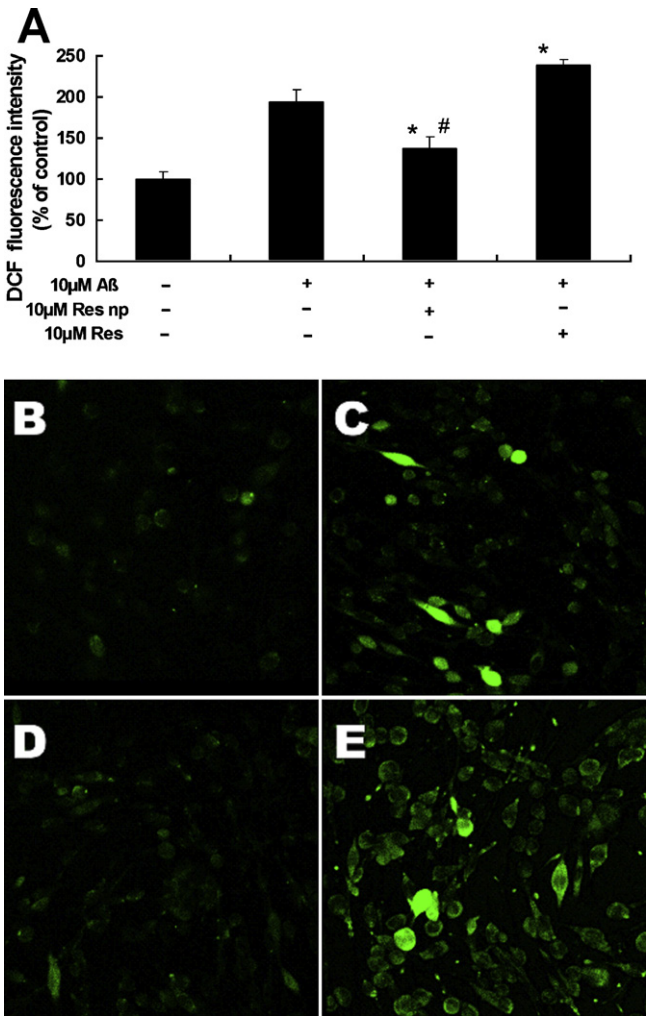

Fig. 5. Effect of 12-h pre-incubation of Res-np or free Res on Aβ-induced ROS accumulation in PC12 cells. (A) Relative DCF fluorescence intensity. Cells were grouped and treated according to the definition in Section 2.7, while ROS accumulation was measured by a fluorescent dye staining method described previously. The obtained values are expressed as a percentage of the negative controls. The higher the fluorescence, the greater the ROS production. 24-h exposure to 10 μM $A\beta_{1-42}$ made an about 100% ($p < 0.05$) increase in ROS level compared to negative control, which was attenuated by pre-incubation of 10 μM Res-np for 12 h ($p < 0.05$). However, pre-incubation of 10 μM free Res for 12 h did not attenuate but enhanced ROS level of Aβ-treated PC12 cells ($p < 0.05$). *Significantly different from the positive control ($p < 0.05$); #significantly different from the groups pretreated with free Res ($p < 0.05$). Values represent mean ± S.D. ($n = 3$). The typical images of the ROS production of PC12 cells were made visible under fluorescent microscope basing on green DCF fluorescence. The brighter the fluorescence, the greater the ROS production. (B) A typical image showing faint ROS production of the negative control. (C) A typical image showing moderate ROS production of the positive control. (D) A typical image showing attenuated ROS production of cells exposed to 10 μM $A\beta_{1-42}$ after 12-h pre-incubation of 10 μM Res-np. (E) A typical image showing great ROS production of cells exposed to 10 μM $A\beta_{1-42}$ after 12-h pre-incubation of 10 μM free Res.

of 10 μM Res-np for 12 h ($p < 0.05$). However, pre-incubation of 10 μM free Res for 12 h did not attenuate but enhanced ROS level of Aβ-treated PC12 cells ($p < 0.05$) (Fig. 5A).

According to DCF fluorescence (green), the typical ROS production of PC12 cells was made visible in Fig. 5B–E under fluorescent microscope. The brighter the fluorescence, the greater the ROS production. To describe briefly, Fig. 5B shows the ROS production of cells without testing drugs nor Aβ exposure (as negative control). Fig. 5C represents the elevated ROS production of cells with Aβ exposure omitting pretreatment of testing drug (as positive control). Fig. 5D and E indicates the attenuated ROS production of cells with pre-incubation of 10 μM Res-np and the enhanced ROS production of cells with pre-incubation of 10 μM Res in the presence of 10 μM $A\beta_{1-42}$, respectively.

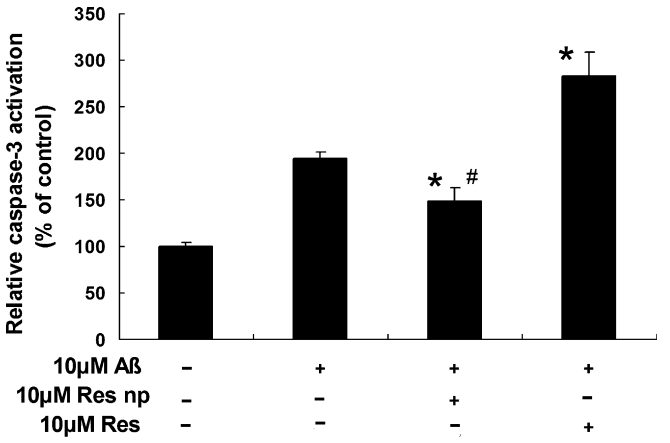

Fig. 6. Effect of 12-h pre-incubation of Res-np or free Res on Aβ-induced caspase-3 activation in PC12 cells.
Cells were grouped and treated according to the definition in Section 2.7, while caspase-3 activation was measured by caspase colorimetric protease assay kit. Caspase-3 activity was shown as the percentage of negative control. The level of activated caspase-3 was significantly increased to 194% ($p < 0.05$, compared to negative control) following exposure to 10 μM $A\beta_{1-42}$ for 24 h, and this elevation of caspase-3 activity was attenuated by 12-h pre-incubation of 10 μM Res-np ($p < 0.05$). Unfortunately, 12-h pretreatment with 10 μM free Res enhanced caspase-3 activity ($p < 0.05$). *Significantly different from the group treated with $A\beta_{1-42}$ alone ($p < 0.05$); #significantly different from the group pretreated with free Res ($p < 0.05$). Values represent mean ± S.D. ($n = 3$).

3.6. Controlled release of resveratrol attenuated caspase-3 activation in Aβ-treated PC12 cells

The activity of caspase-3 was used as a marker of apoptosis in the Aβ-treated PC12 cells (Jang et al., 2007). The level of activated caspase-3 was significantly increased to 194% ($p < 0.05$, compared to negative control) following exposure to 10 μM $A\beta_{1-42}$ for 24 h, and this elevation of caspase-3 activity was attenuated by 12-h pre-incubation of 10 μM Res-np ($p < 0.05$) but enhanced by 12-h pretreatment of 10 μM free Res ($p < 0.05$) (Fig. 6).

4. Discussion

$A\beta_{1-42}$, one of the Aβ variants, has been considered as the main toxic Aβ variant, since it was found richly existing in the senile plaque of AD brain (Perl, 2000). Aβ peptide does harm to neurons through different mechanisms such as ROS generation, mitochondrial dysfunction and apoptosis (Butterfield and Lauderback, 2002; Small et al., 2001). Jang and Surh (2003) and Jang et al. (2007) had reported that $A\beta_{1-42}$ inhibited the viability of PC12 cells in a dose dependent manner and that 2-h pre-incubation of resveratrol at the concentration lower than 25 μM provided protection to PC12 cells against 10 μM $A\beta_{1-42}$ exposure as an antioxidant. For the truth that AD progresses during a long span of time and that resveratrol has been explored as a cytotoxicity agent, it is necessary to evaluate the long-term effect of resveratrol on cells. It had been reported that several hours' incubation of resveratrol showed no cytotoxicity to PC12 cells at the concentration lower than 50 μM (Jang and Surh, 2003; Jang et al., 2007). However, long-term (48 h) treatment with resveratrol showed cytotoxicity to PC12 cells in the current study, even at the concentrations of 5 and 10 μM. Therefore, a longer duration of pre-incubation with resveratrol was further applied to evaluate the effect of it on Aβ protection. Unfortunately, despite the concentrations of Res administrated to the cell culture were at 5 and 10 μM, Res did not protect cells against 10 μM $A\beta_{1-42}$, but

even synergistically inhibit cell viability. Together with the facts that Res either acts as an antioxidant or a pro-oxidant (Alarcón de la Lastra and Villegas, 2007) and that administration of high doses of Res to rats promotes death signals (Jocelyn et al., 2008), these results suggest that the application of Res as a prevention of AD may not be generally applied before further studies are conducted to evaluate its safety and feasibility both in vitro and in vivo. The potential beneficial effects of natural antioxidants cannot justify the risk of severe side effects (Mancuso et al., 2007).

In most experiments resveratrol has been used in a free form dissolved in different organic solvents (i.e., DMSO, acetone, and ethanol) that are not suitable for drug delivery (Athar et al., 2007; Juan et al., 2008; Jocelyn et al., 2008; Jang et al., 2007). Moreover, due to the low bioavailability of trans-resveratrol in animals and humans (Espín et al., 2007) and its poor water-solubility, the reportedly protection concentration range (5–25 μM) cannot be achieved in plasma by common administration. The antioxidant role of resveratrol seems to be considerably diluted in vivo (Anekonda, 2006). By passive and active targeting technologies, researchers have made controlled release drug-loaded nanoparticles across blood–brain barrier (BBB) so as to elevate drug concentration in brain (Kabanov and Gendelman, 2007). Additionally, intracranial implantation of polymeric delivery system has been well explored to treat neurodegenerative diseases (Popovic and Brundin, 2006). The hydrophobicity of Res makes it possible to develop Res-loaded nanocarrier basing on polymeric micelles. Here we developed resveratrol-loaded PCL–PEG nanoparticles by amphiphilic copolymer-based self-assembling technology. It was suggested by the characteristic analysis that Res could be effectively incorporated into the core–shell structure nanoparticles to act as a water-soluble controlled release drug delivery system for therapy. These resveratrol-loaded PCL–PEG nanoparticles were easy to disperse in water even at the equivalent concentration of 50 μM resveratrol (data not shown). First of all, it is unclear whether or not does this kind of Res-loaded nanoparticles protect cells against Aβ toxicity effectively and safely. Then, a predetermined amount of Res-loaded nanoparticles (Res-np) was dispersed into culture media to evaluate their effect on cell viability and Aβ protection.

Nanoparticles containing equivalent doses of Res (5 and 10 μM) were non-toxic to PC12 cells after 48-h incubation. Resveratrol has extremely interesting dual actions, which might depend on cell type, concentration used, duration of contact, and special settings faced by cells (Juan et al., 2008; Caddeo et al., 2008; Alarcón de la Lastra and Villegas, 2007). In the present study, 10 μM $A\beta_{1-42}$ was enough to inhibit PC12 cells and pretreatment with resveratrol for 2 h could protect PC12 cells against the cytotoxicity of 10 μM $A\beta_{1-42}$ in a dose dependent manner at the concentration range of 1–10 μM (Fig. 4B). At the same concentration range, an overnight (12 h) pretreatment with free resveratrol did not protect PC12 cells against the cytotoxicity of 10 μM $A\beta_{1-42}$ or further inhibit the viability of Aβ-treated PC12 cells. However, overnight (12 h) pretreatment with Res-loaded nanoparticles reversed to protect PC12 cells against the toxicity of 10 μM $A\beta_{1-42}$ in a dose dependent manner. These results are similar with another report that controlled release of resveratrol from liposomes can reverse the toxicity of free resveratrol and protect cells against UV-B radiation (Caddeo et al., 2008).

Reactive oxygen species (ROS), the markers of oxidative stress, are removed by enzymatic and non-enzymatic mechanisms that scavenge the radicals to maintain a redox balance (Alarcón de la Lastra and Villegas, 2007). Resveratrol has two phenol groups. With its polyphenolic structure, trans-resveratrol acts as an antioxidant by scavenging ROS (Leonard et al., 2003). However, the excessive lipophilic Res may accumulate in the cell membrane to peroxidize polyunsaturated fatty acid in membrane (Athar et al., 2007; Szende et al., 2000; Sotomatsu et al., 1990; Rice-Evans and Burdon, 1993; Galati et al., 2002; Minotti and Aust, 1989) and generate radicals through the Fenton reaction producing hydroxyl radicals and iron species (Yamazaki and Piette, 1990), which co-oxidizes cellular glutathione or NADH, accompanied by ROS formation (Galati et al., 2002). Due to the endocytosis of these stable drug-loaded biodegradable nanoparticles which end up through an endosome–lysosome pathway (Davda and Labhasetwar, 2002; Shenoy et al., 2005), Res may be partly avoided to accumulate in cell membrane and readily internalized. Slow drug release from the core–shell nanoparticles avoids sudden and massive elevation of intracellular Res. Thus, suitable intracellular concentration of Res slowly releasing from nanoparticles provides protection to cells. This effect enhances with the release of resveratrol from the nanoparticles, so that Res-np acts as antioxidant to protect $A\beta_{1-42}$-treated cells against oxidative stress by scavenge ROS (Caddeo et al., 2008; Shenoy et al., 2005). All the above could interpret the results that long-term treatment of 10 μM free Res was harmful and not able to protect PC12 cells against 10 μM $A\beta_{1-42}$, while the controlled release dosage form of resveratrol might effectively attenuate the oxidative stress of PC12 cells exposed to 10 μM $A\beta_{1-42}$ without long-term cytotoxicity simultaneously.

Plenty of researchers have shown that intracellular ROS accumulation can peroxidize membrane lipids, proteins and DNA and can activate caspase-3, all of which finally lead to apoptosis. This procedure is critical in Aβ-induced cell injury (Jang and Surh, 2003; Savaskan et al., 2003). The characteristic features of apoptosis such as decreased cell viability and caspase-3 activation (Jang et al., 2007) were investigated in Aβ-treated PC12 cells in this study. The activation of caspase-3 and decreased cell viability induced by 10 μM $A\beta_{1-42}$ were reversed by addition of 10 μM Res-loaded nanoparticles but aggravated by 10 μM free Res, which consisted with the synchronic changes of ROS accumulation, respectively.

5. Conclusion

In the current study, 48-h incubation of 5 or 10 μM free resveratrol exhibited toxicity to PC12 cells and 12-h pretreatment with these concentrations of resveratrol did not protect PC12 cells against 10 μM $A\beta_{1-42}$-induced oxidative stress. Successful development of Res-loaded nanoparticles made it possible to protect cells against Aβ-induced damage by attenuating oxidative stress and affecting the apoptosis without long-term cytotoxicity. It is hypothesized that administering resveratrol via injectable and biodegradable drug delivery system might be a potential therapeutic tool. However, it is undoubted that intensive researches are expected to assess the feasibility and advantages of in vivo applications of antioxidant by polymeric nanotechnology.

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