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Prostate Apoptosis Response-4 Involved in the Protective Effect of Salvianolic Acid B Against Amyloid β Peptide-Induced Damage in PC12 Cells

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ABSTRACT—To observe the effect of salvianolic acid-B (SalB) against the cytoxicity of amyloid β peptide (A-beta)(25 – 35) to PC12 cells, the cells were incubated with A-beta, and the cytoxicity was investigated by MTT, flow cytometry and a cell free apoptotic system. The expression of prostate apoptotic response-4 (Par-4) was detected by Western blot. Aged A-beta 10 μmol/L significantly inhibited the MTT reduction of PC12 cells, SalB 1 μmol/L inhibited the toxicity induced by A-beta. In flow cytometric analysis, PC12 cells treated with A-beta exhibited degraded DNA content characteristic of apoptosis cells (1.53% vs 19.9%). PC12 cells pretreated with SalB (10 nmol/L, 100 nmol/L, 1 μmol/L) manifested relatively low proportion of apoptosis (15.7%, 13.5%, 11.8%, respectively). SalB (10 nmol/L – 1 μmol/L) when added at the beginning of the cell free apoptotic reaction had no apparent effect on the nuclei apoptosis. Pretreatment of PC12 cells with SalB largely prevented the increase in Par-4 expression of the cells when they were exposed to A-beta. The results suggest that Par-4 is involved in the protective effect of SalB against A-beta-induced damage in PC12 cells.

Keywords: Salvianolic acid B, Amyloid β peptide, Prostate apoptosis response-4, PC12 cell

Accumulation of β-amyloid peptide (A-beta) in the brain is a defining feature of Alzheimer disease (AD) (1). The fact that A-beta can be neurotoxic in vitro and in vivo suggests that this protein may be actively involved in the neuronal degeneration that occurs in AD (2 – 4). The fibril formation and aggregation of A-beta was now considered to be a necessary step for its neurotoxicity. Extensive studies have shown that A-beta induced neurotoxicity in multiple cell types may be mediated by several different mechanisms and free radicals involve in the neurotoxic effect (5 – 9). Recently, more attention have been paid to prostate apoptosis response-4 (Par-4), an apoptotic factor first identified in tumor cells (10). Guo et al. (11) suggested that Par-4 is involved in the pathogenesis of AD. Culmsee et al. (12) and Dhillon et al. (13) demonstrated that early up-regulation of Par-4 plays a pivotal role in ischemic neuronal death in animal models of stroke and traumatic brain injury. At present, the initial importance of Par-4 in neurodegeneration was generally accepted (14).

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Salvianolic acid-B (SalB) is one of the water-soluble components isolated from the traditional Chinese drug *Radix Salviae Miltiorrhizae*. Our previous study found that SalB had strong anti-oxidant activity (15) and could inhibit the fibril formation and aggregation of A-beta (16). The present study was designed to investigate the effect of SalB on PC12 cells treated with aged A-beta.

MATERIALS AND METHODS

Drugs and reagents

SalB (>90%) was supplied by the Department of Phytochemistry of our institute, and its molecular weight is 718 ($C_{36}H_{30}O_{16}$) (Fig. 1). A-beta(25 – 35) was purchased from Sigma (St. Louis, MO, USA). Aprotinin (Trasylol) was obtained from Boehringer (Mannheim, Germany). Mouse anti-Par-4 antibody and alkaline phosphatase horse anti-mouse antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture

PC12 cells were obtained from the Shanghai Institute of

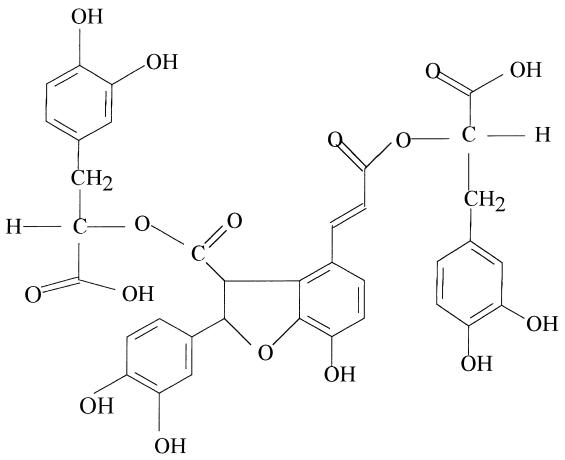

Fig. 1. Structure of salvianolic acid B.

Cell Biology, Chinese Academy of Sciences. The cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 10% horse serum, at 37°C, in an atmosphere containing 5% CO_2.

MTT reduction

Cytotoxic effect of A-beta was assessed by measuring cellular redox activity with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (6). A-beta was dissolved in sterilized 0.1 mol/L sodium phosphate buffer (pH 7.4) to a concentration of 1 g/L and then incubated at 37°C for 7 days for aging. PC12 cells were plated at a density of 5,000 cells/100 μL per well in 96-well tissue culture plates. After 1-h incubation with SalB or phosphate-buffered saline (PBS, pH 7.4), A-beta solution was added to the wells to a final concentration of 10 μmol/L. Following 48-h incubation at 37°C, MTT reduction was measured.

Flow cytometric analysis

PC12 cells were plated at a density of 50,000 cells/ml per well in 6-well tissue culture plates. The next day, SalB or PBS was added to the wells and incubated at 37°C for 1 h, and then A-beta was added to the wells to a final concentration of 10 μmol/L. Following a 6-day incubation at 37°C, the cells were harvested, stained with propidium iodide (PI), and analyzed in a flow cytometer (Coulter EpicsXL; Beckman Coulter, Fullerton, CA, USA) excited at 488-nm wavelength and collected through a 570-nm BP filter. Ten thousand cells were counted per sample.

Cell free apoptosis

Preparation of cytoplasmic extracts: Cytoplasmic extracts were isolated from PC12 cells using a protocol similar to that described by Ellerby et al. (17). Briefly, PC12 cells (6×10^5 cell/mL) were treated with 10 μmol/L A-beta or PBS for 72 h. The cells were harvested by centrifugation, washed with PBS, and then resuspended in 1 volume of hypotonic extraction buffer (HEB; containing 10 mmol/L HEPES, pH 7.4, 50 mmol/L KCl, 5 mmol/L EGTA, 2 mmol/L $MgCl_2$, 1 mmol/L DTT, 0.1 mmol/L PMSF). The cells were allowed to swell for 30 min at 0°C, and then they were transferred to a Dounce homogenizer and lysed with 50 gentle strokes with a B-type pestle at 0°C. The desired extent of lysis (>90%) was monitored under a microscope by trypan blue staining. The cell lysate was transferred to a 1.5-mL Eppendorf tube and centrifuged at 16,000 × *g* (4°C) for 30 min. The clarified supernatant was stored at –20°C and used within 2 h.

Preparation of nuclei: Nuclei were isolated from PC12 cells using a protocol similar to that described by Mattson et al. (18). Briefly, cells were harvested by centrifugation, washed with PBS, and resuspended in 5 vol of nuclei isolation buffer (10 mmol/L HEPES, pH 7.4, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L DTT, 1 mmol/L PMSF, 0.01 mmol/L cytochalasin B). Cells were allowed to swell for 30 min at 0°C. Then the cells were transferred to a Dounce homogenizer and lysed with 10 gentle strokes of a B-type pestle at 0°C. The homogenate was layered atop a solution of 30% sucrose in nuclei isolation buffer and centrifuged at 840 × *g* for 10 min. Then the nuclei were washed, resuspended in Locke's solution in a density of 1.5×10^8 nuclei/mL, and stored at 4°C. The nuclei were used within 30 min.

Activation of cell-free apoptosis: The cell free apoptosis reaction system contained 40 μL of normal or apoptotic cytoplasmic extract, 10 μL nuclei (1.5×10^6 nuclei), and 5 μL SalB or PBS. The reaction was activated by incubating at 25°C for 12 h. Nuclei were then stained with PI (10 μmol/L) and imaged with a confocal laser scanning microscope. The percentage of nuclei with apoptotic morphologies (condensed and fragmented chromatin) was determined.

Par-4 expression

PC12 cells were plated at a density of 100,000 cells/mL per well in 6-well tissue culture plates. SalB or PBS was added to the wells and incubated at 37°C for 1 h, then A-beta was added to the wells to a final concentration of 10 μmol/L. Following a 24-h incubation at 37°C, the cells were lysed with RIPA buffer (0.01 mol/L sodium phosphate buffer, pH 7.2, 1% NP-40, 0.1% SDS, 1% deoxycholate sodium, 150 mmol/L NaCl, 2 mmol/L EDTA, 100 mg /L PMSF, 2 mg/L aprotinin). The cell lysate was centrifuged at 15,000 × *g* (4°C) for 20 min. The supernatant was used for protein detection (19) and Western blotting. A 50-μg sample of solubilized protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel) and transferred to a polyvinylidene difluoride (PVDF)

membrane. The membrane was incubated with Par-4 antibody (1:1000) for 2 h and alkaline phosphatase horse antimouse secondary antibody (1:1000) for 2 h. Immunoreactive protein was visualized using a NBT/BCIP kit (Zhong Shan Biotechnology Co., Ltd., Beijing, China). A densitometry analysis was done for the reuslts.

Statistical analysis

Data are presented as means ± S.D. and significance was determined by Student's *t*-test. A value of $P<0.05$ was considered to be significant.

RESULTS

MTT reduction

Aged A-beta at 10 μmol/L significantly inhibited the MTT reduction of PC12 cells, which was 85% of the control following a 48-h incubation. SalB at 1 μmol/L significantly alleviated the inhibitory effect of A-beta, with the MTT reduction of 92.7%; and SalB at 10 and 100 nmol/L manifested a similar effect (87.5% and 89.7%, respectively), although no significant difference was observed (Fig. 2).

Flow cytometric analysis

In flow cytometric analysis, as shown in Fig. 2, PC12 cells treated with A-beta exhibited obvious degraded DNA content characteristic of apoptosis cells (1.53% vs 19.9%). PC12 cells pretreated with 10 nmol/L, 100 nmol/L and 1 μmol/L SalB manifested relatively low proportion of apoptosis (15.7%, 13.5%, 11.8%, respectively), when the cells were treated with A-beta (Fig. 3).

Cell free apoptosis

Few nuclei exhibited apoptotic morphologies when they

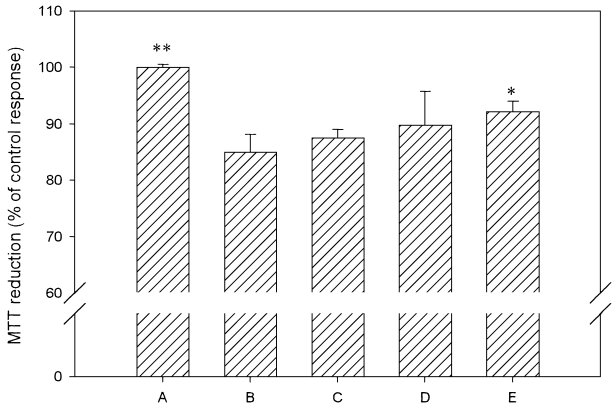

Fig. 2. SalB alleviated the inhibitory effect of A-beta(25 – 35) on the MTT reduction of PC12 cells. A: Vehicle, B: A-beta, C: A-beta + 10 nmol/L SalB, D: A-beta + 100 nmol/L SalB, E: A-beta + 1 μmol /L SalB. *$P<0.05$, **$P<0.01$ vs A-beta (B). n = 3, means ± S.D.

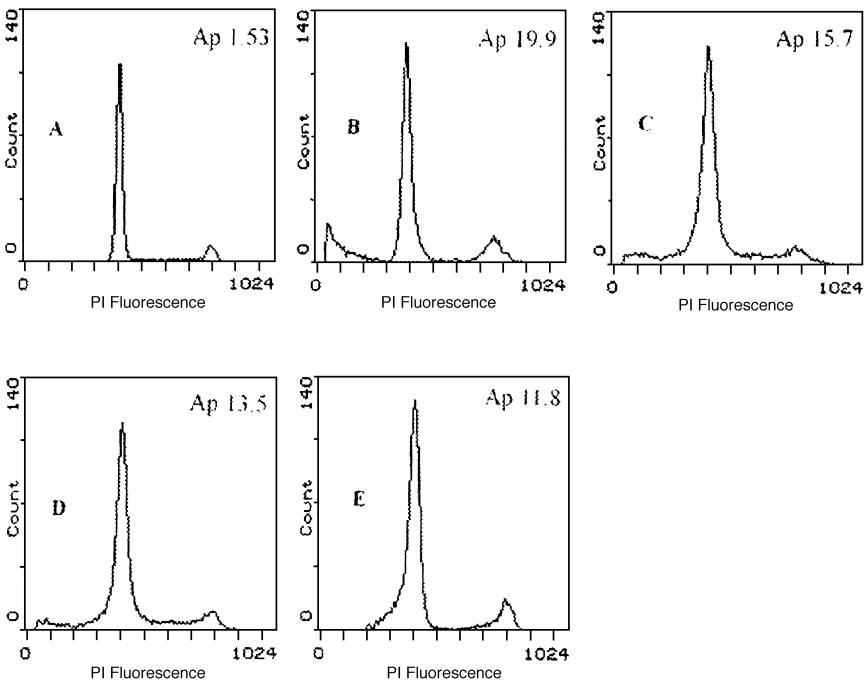

Fig. 3. SalB inhibited the apoptosis of PC12 cells induced by A-beta. Cells were plated in 6-well tissue culture plates. The next day SalB or PBS was added to the wells and incubated at 37°C for 1 h, and then A-beta(25 – 35) was added to the wells to a final concentration of 10 μmol/L. Following a 6-day incubation, the cells were analyzed in a flow cytometer. A: Control, B: A-beta, C: A-beta + 10 nmol/L SalB, D: A-beta + 100 nmol/L SalB, E: A-beta + 1 μmol/L SalB. Ap: Apoptosis percentage.

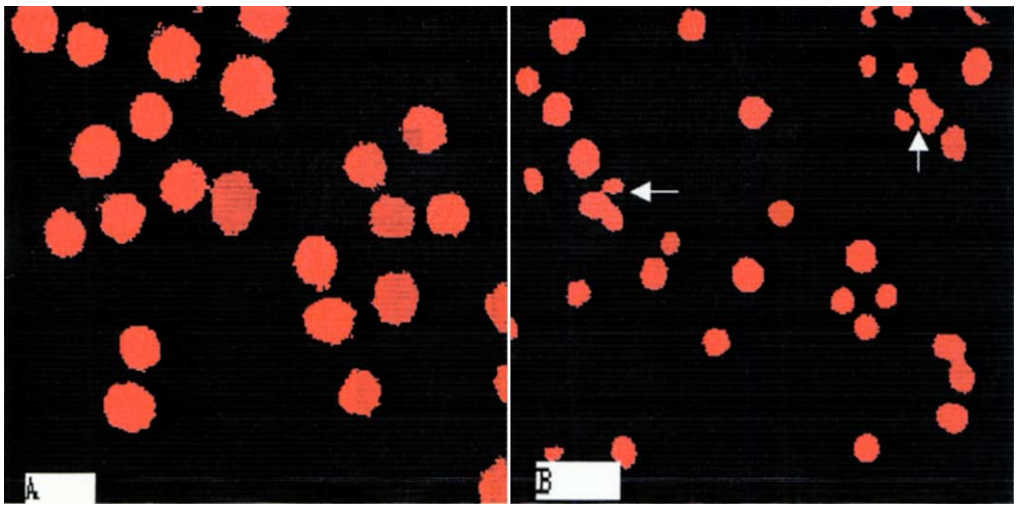

Fig. 4. Cell free apoptosis induced by A-beta. The cells were treated with PBS or A-beta(25 – 35) for 72 h. The cytoplasmic extract or nuclei was extracted. The cell-free apoptosis reaction was activated by incubating at 25°C for 12 h. Nuclei were then stained with PI (10 μmol/L) and imaged with a confocal laser scanning microscope. The percentage of nuclei with apoptotic morphologies (fragmented chromatin and condensed) was determined. A: nuclei treated with normal extract; B: nuclei treated with A-beta extract, the arrows indicate typical apoptotic nuclei.

were exposed to cytosolic extract from PBS treated cells (Fig. 4A). Exposure of isolated nuclei to cytosolic extracts from A-beta treated cells resulted in a relative high increase in the percentage of nuclei exhibiting apoptotic morphologies (Fig. 4B). SalB (10 nmol/L – 1 μmol/L) when added at the beginning of the cell free apoptosis reaction has no apparent effect on the nuclei apoptosis (Table 1).

Par-4 expression

Western blot analysis showed that Par-4 is present in PC12 cells. Exposure of the cells to A-beta for 24 h resulted in an increase of Par-4 expression (density, 375506 vs 114566). Pretreatment of PC12 cells with SalB 100 nmol /L and 1 μmol/L largely prevented the increase in Par-4 expression of the cells (density, 101143 and 193392, respectively) (Fig. 5).

Table 1. Effect of SalB on cell free apoptosis induced by A-beta(25 – 35)

Group	Apoptotic nuclei (%)
Vehicle	3.3 ± 1.0
A-beta	20.0 ± 1.8**
A-beta + 1 μmol/L SalB	18.4 ± 3.3**
A-beta + 100 nmol/L SalB	20.5 ± 3.6**
A-beta + 10 nmol/L SalB	20.2 ± 2.0**

Cytoplasmic extracts were isolated from PC12 cells treated with 10 μmol/L A-beta or PBS for 72 h. Nuclei were isolated from PC12 cells. The cell free apoptosis reaction was activated by incubating nuclei and cytoplasmic extracts at 25°C for 12 h. Nuclei were then stained with PI (10 μmol/L) and imaged with a confocal laser scanning microscope. The percentage of nuclei with apoptotic morphologies (condensed and fragmented chromatin) was determined. **P<0.01 vs vehicle. n = 3, means ± S.D.

DISCUSSION

According to our previous study (16), incubating A-beta peptide for 7 days can result in a thorough aggregation and fibril formation for the peptide. Here in the present study, we used the aged peptide on PC12 cells and found SalB protected the cells from A-beta induced damage. Since the cytoxicity of A-beta has been shown to be associated with

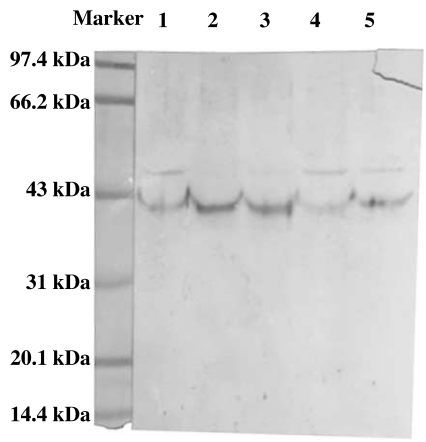

Fig. 5. SalB inhibited Par-4 expression in PC12 cells induced by A-beta. PC12 cells were treated with 10 μmol/L A-beta. Following a 24-h incubation at 37°C, the cells were lysed with RIPA buffer. Western blot was performed and a densitometry analysis was done for the results. 1: Control, 2: A-beta, 3: A-beta + 10 nmol/L SalB, 4: A-beta + 100 nmol/L SalB, 5: A-beta + 1 μmol/L SalB.

its fibrillar forms (9, 20), inhibition of the fibril formation is viewed as a possible method of slowing disease progression in AD. We previously demonstrated that SalB could inhibit the fibril formation and aggregation of A-beta (16); and furthermore, the present results that SalB protected PC12 cells from aged A-beta induced damage suggest that SalB can conduct its protective effect by mechanisms relatively down-stream the fibril formation site.

When treated with A-beta, cells may involved in death by several mechanisms (8, 21, 22), such as calcium, reactive oxygen species (ROS), and caspase-like proteinase that can result in the apoptotic cleavage of nuclei and finally result in the death of the cells. The present result that SalB has a protective effect against the cytoxicity of aged A-beta gives rise to another argument. Does SalB exert its effect by directly contradicting apoptotic effectors (such as caspase family, ROS) that exist in the cytoplasm due to the insults or by indirectly inhibiting the effects of the apoptotic effectors? Hence we used a cell-free apoptotic system in this study. However, we found no protective effect of SalB against apoptotic cytoplasmic extracts-induced nuclei cleavage. The result may indicate that SalB protects the cells at site more up-stream these apoptotic effectors.

There are several genes up-stream the apoptotic effectors that can trigger the apoptotic process (23). The up-stream regulators in the cells are activated first when treated with apoptotic stimuli, and then an activation of apoptotic effectors or inhibition of anti-apoptotic factors followed (14). Finally the cells may undergo apoptosis or necrosis. Par-4 is one of the up-stream regulators. It is up-stream of the caspase family and exerts its apoptotic effect by interacting with other proteins (14, 24) such as PKCζ and Bcl-2. By interacting with this protein, Par-4 results in suppression of the anti-apoptitic system. Guo et al. (11) found that Par-4 mRNA and protein increased in AD brain and overexpression of Par-4 in PC12 cells resulted in an increase of apoptosis when the cells were treated with A-beta. The author suggests that Par-4 is involved in the initiation of AD. In this study we found that incubation of PC12 cells with A-beta resulted in a significant decrease in the MTT reduction, the same time Par-4 expression in the cells obviously increased. The result further indicated the significance of Par-4 in A-beta-induced cell damage. SalB protected PC12 cells against A-beta-induced damage in this study in a concentration-dependent manner. A similar result was observed in Par-4 expression when the cells were treated with SalB. We therefore suggest that SalB conducted its protective effect partially by inhibiting the A-beta-induced Par-4 expression. However, how SalB inhibited the expression of Par-4 should be studied further.

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