Effects of thymosin β4 on oxygen-glucose deprivation and reoxygenation-induced injury

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Received May 12, 2017; Accepted January 4, 2018

DOI: 10.3892/ijmm.2018.3369

Abstract. Cerebral ischemia causes severe brain injury and results in selective neuronal death through programmed cell death mechanisms, including apoptosis and autophagy. Minimizing neuronal injury has been considered a hot topic among clinicians. The present study elucidated the effect of thymosin β4 (Tβ4) on neuronal death induced by cerebral ischemia/reperfusion in PC12 cells that were subjected to oxygen-glucose deprivation and reoxygenation (OGD/R). The survival, apoptotic and autophagy rates of PC12 cells were investigated. Tβ4 pre-conditioning prior to OGD/R was performed to evaluate PC12-cell viability and the protective mechanisms of Tβ4. Tβ4 significantly increased cell survival after OGD/R. Tβ4 inhibited the release of lactate dehydrogenase, downregulated malondialdehyde and upregulated the activities of glutathione peroxidase and superoxide dismutase. In addition, Tβ4 attenuated OGD/R-associated decreases in the expression of P62 and the anti-apoptotic protein B-cell lymphoma-2, as well as the upregulation of autophagy mediators, including autophagy-related protein-5 and the ratio of microtubule-associated protein 1 light chain 3 (LC3) II vs. LC3 I. These results suggested that Tβ4 effectively inhibits cell apoptosis and autophagy induced by OGD/R. To the best of our knowledge, the present study was the first to report on the antioxidant, anti-apoptotic and anti-autophagic effects of Tβ4 in neuronal-like PC12 cells. These results suggested that Tβ4 may be explored as a potential treatment for cerebral ischemia.

Introduction

Cerebral stroke is a common neurological event (1). Cerebral ischemia/reperfusion may cause oxygen and nutrient deprivation (2) and induce neuronal injury (3). Minimizing neuronal injury has been considered a hot topic among investigators and clinicians.

Thymosin β4 (Tβ4) is a pleiotropic polypeptide (4). It sequesters G-actin and is necessary for cell motility and organogenesis. Previous studies have indicated that Tβ4 promotes tissue repair (5,6). The safety, tolerability and efficacy of Tβ4 are being evaluated in clinical applications (7,8).

Tβ4 expression in developing brain tissue correlates with neuronal migration and neurite extension. A series of studies have suggested that Tβ4 may also have neuroprotective effects. Choi *et al* (9) reported that Tβ4 suppressed staurosporine-induced neuronal apoptosis *in vitro*, Popoli *et al* (10) indicated that Tβ4 attenuated glutamate-induced toxicity, and Morris *et al* (11) and Xiong *et al* (12) demonstrated that Tβ4 improves the outcome for rats subjected to acute stroke or traumatic brain injury. Tβ4 treatment also induced oligodendrocyte differentiation and myelin gene expression (13), but the direct effect of Tβ4 on neurons has remained to be fully elucidated.

As mentioned above, cerebral ischemia/reperfusion causes severe brain injury and results in neuronal death through programmed cell death mechanisms (14), including necrosis, apoptosis and autophagy; the latter two are more commonly observed in *in vitro* experiments (15).

Autophagy is an intracellular bulk degradation process that is essential to maintain cellular metabolism and homeostasis (16). Vast evidence indicates that excessive autophagic activity triggers autophagic cell death in numerous diseases (17), including neurodegenerative diseases (18) and cerebral ischemia (19,20).

However, the role of Tβ4 in autophagy and apoptosis still requires clarification. In the present study, PC12 cells were used in a model of oxygen-glucose deprivation and reoxygenation (OGD/R) (21) in order to investigate the effect of Tβ4 on neural cells subjected to cerebral ischemia/reperfusion injury.

Materials and methods

Materials. Tβ4 was purchased from ProSpec (Ness-Ziona, Israel) and dissolved in distilled water. The PC12 pheochromocytoma cell line was obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). The

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Key words: thymosin β4, oxygen-glucose deprivation and reoxygenation, apoptosis, autophagy

reagents for cell culture, including Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS), were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). MTT was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The assay kits for the determination of superoxide dismutase (SOD) activity (cat. no. S0107) and malondialdehyde (MDA) content (cat. no. S0131) were purchased from Beyotime Institute of Biotechnology (Haimen, China). The assay kit for the determination of glutathioneperoxidase (GSH-Px) activity (cat. no. A005) was purchased from Jiancheng (Nanjing, China). TRIzol for RNA isolation and the Power SYBR® Master Mix were from Invitrogen (Thermo Fisher Scientific, Inc.). Primary mouse monoclonal antibodies against B-cell lymphoma 2 (Bcl-2; cat. no. sc-7382), Beclin-1 (cat. no. sc-48341), microtubule-associated protein 1 light chain 3 I/II (LC3I/II; cat. no. sc-398822) and β-actin (cat. no. sc-130300) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Primary antibodies for active caspase-3 (cat. no. ab2302) and P62 (cat. no. ab56416) were purchased from Abcam (Cambridge, MA, USA). Secondary antibodies (cat. no. 31430) and a lactase dehydrogenase (LDH) cytotoxicity assay kit (cat. no. 88953) were from Pierce (Thermo Fisher Scientific, Inc.). An Annexin V-fluorescein isothiocyanate (FITC) cell apoptosis assay kit (cat. no. C1063) was purchased from Beyotime Institute of Biotechnology.

Cell culture and the OGD/R cell model. The PC12 cells were cultured in DMEM with 10% FBS at 37˚C in a humid atmosphere containing 5% CO_2. Cells were divided into a control group, an OGD/R group and an OGD/R+Tβ4 group (Tβ4 intervention group; 0.1, 1 or 10 mg/l Tβ4 was added). In the OGD/R group, DMEM was replaced with serum-free, glucose-free Earle's buffer supplemented with 10 mmol/l $Na_2S_2O_4$ (Sigma-Aldrich; Merck KGaA), followed by incubation at 37˚C for 4 h in air containing 5% CO_2, and then the $Na_2S_2O_4$ was removed. Subsequent culture in DMEM supplemented with serum and glucose in air containing 5% CO_2 for 2 h was performed. In the control group, the cells were incubated in DMEM in a normoxic atmosphere for the same duration. In the Tβ4 intervention group, the cells were pre-treated with 0.1-10 mg/l Tβ4 for 2 h and cultured in serum-free, glucose-free Earle's buffer supplemented with 10 mmol/l $Na_2S_2O_4$ for 4 h. The $Na_2S_2O_4$ was then removed and the cells were cultured in DMEM supplemented with serum and glucose for a further 2 h.

MTT assay. The PC12 cells from all groups were cultured at 37˚C with 5% CO_2 for 24 h. MTT solution was added to each well, followed by incubation for 4 h at 37˚C. The culture medium was then removed and dimethylsulfoxide was added to each well, followed by incubation with agitation for 10 min. The viability was determined by measuring the optical density (OD) at 562 nm. The cell viability in each group was calculated as a percentage of the control group.

Measurement of LDH release. In brief, after OGD/R, the supernatant of the four groups were assessed using the LDH Cytotoxicity Assay kit. The LDH release, which is associated with cell damage, was measured at 490 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

Lipid peroxidation assay. In brief, after OGD/R, the cells were washed, homogenized and centrifuged at 12,000 x g for 10 min at 4˚C. The MDA content was measured using an MDA assay kit, according to the manufacturer's protocol. The results are expressed in µmol/l.

Analysis of SOD and GSH-Px activity. In brief, after OGD/R, the cells were resuspended and homogenized. The supernatant was collected for further experiments. The activity levels of the intracellular antioxidant enzymes SOD and GSH-Px were measured using commercial assay kits, according to the manufacturer's protocols. SOD and GSH-Px activity was expressed in U/ml.

Flow cytometric analysis. The cells were analyzed by flow cytometry using a BD FACS Aria flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The cells were stained with Annexin V-FITC and propidium iodide using the Annexin V-FITC apoptosis detection kit according to the manufacturer's instructions.

Western blot analysis. Samples were homogenized in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate). Protein concentrations were determined using a BCA kit (Haoji Biotec, Inc., Hangzhou, China). The protein samples (60 µg per lane) were separated using 5-10% SDS-PAGE. Following electrophoresis, separated proteins were transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were then blocked in 5% non-fat milk and probed overnight at 4˚C with the following primary antibodies: Anti-LC3 (1:1,000 dilution), anti-Beclin 1 (1:500 dilution), anti-Bcl-2 (1:500 dilution), anti-cleaved (active) caspase-3 (1:1,000 dilution), anti-P62 (1:500 dilution) and anti-β-actin (1:5,000 dilution). The membranes were then washed 3 times with Tris-buffered saline containing Tween-20 and subsequently incubated with the respective secondary antibodies (1:5,000 dilution) for 1 h at room temperature. The blots were visualized using enhanced chemiluminescence SuperSignal® West Dura Extended Duration Substrate (cat. no. 34075; Pierce; Thermo Fisher Scientific, Inc.) and images were captured on X-ray film (Kodak, Rochester, NY, USA), which was scanned and quantified. The density of bands corresponding to proteins of interest was normalized to the density of the control β-actin band. Five independent experiments were performed using duplicate samples.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from cells using TRIzol reagent, and 5 µg RNA was reverse transcribed into cDNA using a 1st-Strand cDNA Synthesis kit (Haoji Biotec, Inc.) to a final reaction volume of 20 µl (including RT buffer mix, Primer mix and RT Enzyme mix). The RT reaction was as follows: 30˚C for 10 min and cooling on ice and 42˚C for 30 min, according to the manufacturer's protocol. The reaction was terminated by heating at 70˚C for 15 min. Primers for Beclin-1 forward, 5'-GGCAGTGGCGGCTCCTATTC-3' and reverse, 5'-CTGTGAGGACACCCAAGCAAGAC-3'; autophagy-related protein-5 (Atg5) forward, 5'-TCAGCTCTG CCTTGGAACATCA-3' and reverse, 5'-AAGTGAGCCTCA ACTGCATCCTT-3' and control 18S RNA (18S) forward, 5'-GAATTCCCAGTAAGTGCGGGTCATA-3' and reverse, 5'-CGAGGGCCTCACTAAACCATC-3' were designed with Primer Premier 6.0 (Premier Biosoft International, Palo Alto, CA, USA) and Beacon Designer 7.8 (Premier Biosoft International) and synthesized by Sangon Biotech (Shanghai, China). The relative mRNA expression levels were then evaluated by qPCR on an ABI 7300 PCR application (Applied Biosystems; Thermo Fisher Scientific, Inc.) with a Power SYBR® Master mix (Invitrogen; Thermo Fisher Scientific, Inc.). PCR was performed under the following conditions: 95˚C for 1 min, followed by 40 cycles of 95˚C for 10 sec and 64˚C for 25 sec. Rat 18S RNA was used as the reference gene. The $2^{-\Delta\Delta Cq}$ method (22) was used to quantify the mRNA of target genes.

Statistical analysis. Values are expressed as the mean ± standard deviation and were analyzed using Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical analyses were performed using one-way analysis of variance followed by Dunnett's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Tβ4 reduces OGD-induced cell damage. As indicated by the MTT assay, PC12-cell viability in the OGD/R group was reduced by nearly 60%. In the OGD/R+Tβ4 groups, the cells were pre-treated with 0.1, 1 or 10 mg/l Tβ4 for 2 h and then cultured under OGD/R conditions for 6 h. The results indicated that pre-treatment with Tβ4 did not affect the cell viability in the groups that were not exposed to OGD/R, but Tβ4 at the 1 and 10 mg/l concentrations reduced the OGD/R-induced cell death of PC12 cells in a dose-dependent manner (Fig. 1).

Tβ4 reduces OGD-induced LDH leakage. In the OGD/R+Tβ4 groups, 0.1, 1 or 10 mg/l Tβ4 was added to the culture for 2 h and then cultured under OGD/R conditions for 6 h. The results indicated that 10 mg/l Tβ4 effectively suppressed OGD/R-induced LDH release from PC12 cells (Fig. 2).

Tβ4 reduces OGD-induced changes in MDA levels, as well as SOD and GSH-Px activities. As presented in Fig. 3A, the MDA content was significantly increased under OGD/R conditions compared with that in the control group (P<0.05). However, pre-treatment with Tβ4 at the concentrations of 0.1, 1 or 10 mg/l Tβ4 markedly decreased the MDA content by 21.60, 32.4 and 37.60%, respectively, of that in the OGD/R group. The activities of the antioxidant enzymes SOD and GSH-Px under OGD/R conditions were then investigated. As presented in Fig. 3B and C, OGD/R significantly decreased the activities of SOD and GSH-Px compared with those in the control group (P<0.05). However, SOD and GSH-Px activities were significantly increased by pre-treatment with 1 and 10 mg/l Tβ4 in a concentration-dependent manner compared with those in the OGD/R group (P<0.05). These results suggested that Tβ4 attenuated the oxidative damage to cells under OGD/R conditions and maintained the activity of antioxidant enzymes.

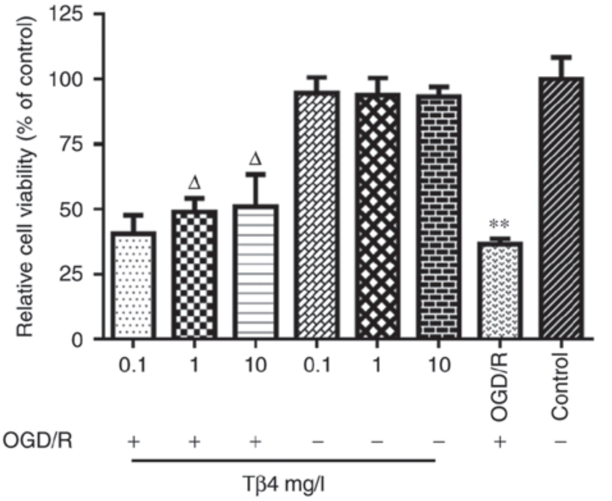

Figure 1. Influence of Tβ4 on OGD/R-induced cell viability. MTT assay of PC12 cells treated with different doses of Tβ4 (n=8). The results indicated that pre-treatment with Tβ4 reduced OGD/R-induced cell death of PC12 cells in a dose-dependent manner, with a marked cytoprotective effect at 1 and 10 mg/l Tβ4. **P<0.01 vs. the control group; $^{\Delta}$P<0.05 vs. the OGD/R group. Tβ4, thymosin β4; OGD/R, oxygen-glucose deprivation and reoxygenation.

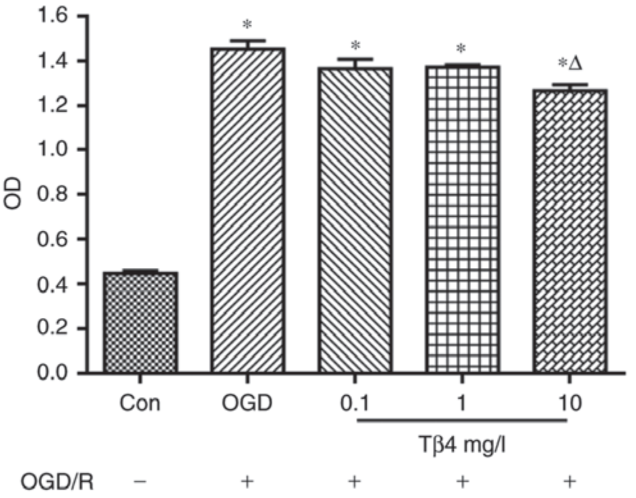

Figure 2. Influence of Tβ4 on OGD/R-induced LDH release. OGD/R-induced LDH leakage from PC12 cells treated with different doses of Tβ4 (0.1, 1 or 10 mg/l). Tβ4 treatment at 10 mg/l effectively suppressed OGD/R-induced LDH release from PC12 cells. *P<0.01 vs. the control group; $^{\Delta}$P<0.05 vs. the OGD/R group (n=8). Tβ4, thymosin β4; OGD/R, oxygen-glucose deprivation and reoxygenation; LDH, lactate dehydrogenase; OD, optical density.

Tβ4 attenuates OGD/R-induced apoptosis. The flow cytometry results indicated that different concentrations of Tβ4 (0.1, 1 or 10 mg/l) significantly increased the cell survival percentage (Q1) after exposure to OGD/R (26.85±0.61, 25.22±0.53 and 24.75±0.50%, respectively, vs. 5.35±0.13% in the OGD/R group) and reduced the percentage of cells in late apoptosis/necrosis (Q2; 6.35±0.34, 5.50±0.23 and 12.11±1.35%, respectively, vs. 21.83±0.57% in the OGD/R group). In addition, the high concentration of Tβ4 reduced the number of early apoptotic cells (Q3) (58.65±0.94 vs. 72.03±1.32% in

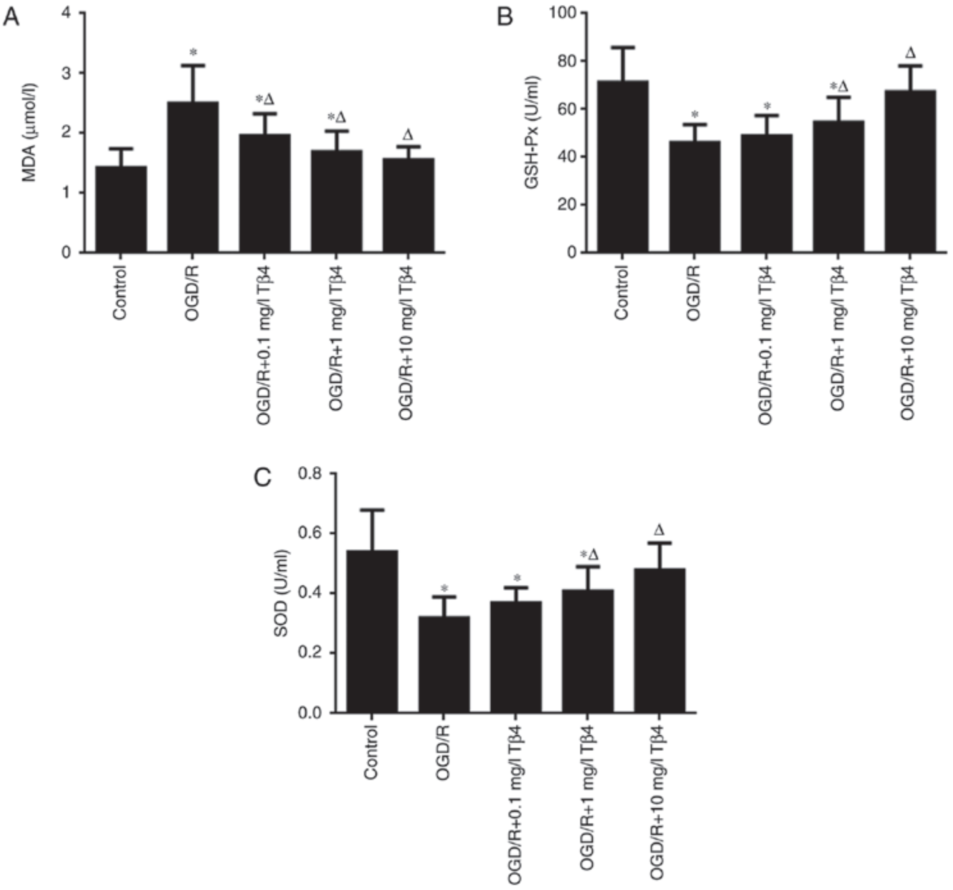

Figure 3. Tβ4 dose-dependently reduces the MDA content and increases the activities of SOD and GSH-Px in cells under OGD/R conditions. Effects of Tβ4 on (A) the MDA content and the activities of (B) GSH-Px and (C) SOD in cells under OGD/R conditions. Pre-treatment with Tβ4 markedly decreased the MDA content and increased SOD and GSH-Px activities compared with those in the OGD/R group (P<0.05). *P<0.05 vs. the control group; $^{\Delta}$P<0.05 vs. the OGD/R group (n=6). Tβ4, thymosin β4; OGD/R, oxygen-glucose deprivation and reoxygenation; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase.

the OGD/R group). These results demonstrated that Tβ4 has an obvious protective effect on PC12 cells against OGD/R damage (Fig. 4).

Tβ4 reduces OGD/R-induced apoptotic signaling. Western blot analysis indicated that the levels of Bcl-2 were decreased in PC12 cells following OGD/R; however, these levels were significantly increased in the presence of Tβ4 compared with those in the OGD/R group (Fig. 5).

Tβ4 reduces OGD/R-induced autophagy-associated gene expression. RT-qPCR analysis demonstrated that following OGD/R, the expression levels of Beclin-1 and Atg-5 were increased compared with those in the control group, and only the Atg-5 levels were significantly decreased in the presence of Tβ4 (1 and 10 mg/l) compared with those in the OGD/R group (Fig. 6).

Tβ4 regulates OGD/R-induced autophagy-associated protein expression. Western blot analysis demonstrated that the levels of Beclin-1 and the ratio of LC3 II vs. LC3 I were increased in PC12 cells subjected to OGD/R, and the ratio of LC3 II vs. LC3 I was significantly inhibited in the presence of Tβ4 (10 mg/l). The levels of P62 were decreased in the OGD/R group; however, these levels were significantly increased in the presence of Tβ4 (10 mg/l) compared with those in the OGD/R group (Fig. 7).

Discussion

Cerebral ischemia induces neuronal cell death through mechanisms including apoptosis and autophagy. In the present study, the MTT assay and flow cytometric analysis indicated that pre-treatment with Tβ4 reduced OGD/R-induced PC12 cell death. Tβ4 also effectively suppressed OGD/R-induced LDH release, decreased the MDA content and significantly increased SOD and GSH-Px activities, which suggests that Tβ4 attenuated the oxidative damage to PC12 cells following OGD/R. Western blot analysis revealed that Tβ4 reduced the OGD/R-induced decreases in Bcl-2 expression, which suggests that Tβ4 has an obvious anti-apoptotic effect on PC12 cells subjected to OGD/R.

Distinct from apoptosis, autophagy is a type of programmed cell death that is mediated by self-digestion (23) and degradation of organelles (24). Therefore, the role of autophagy in various diseases has become a hot research topic (25).

Excessive autophagic activity may lead to cell death in acute neurological disorders, including cerebral ischemia (26). Xie *et al* (27) reported that selective deletion of Atg-7 prevented

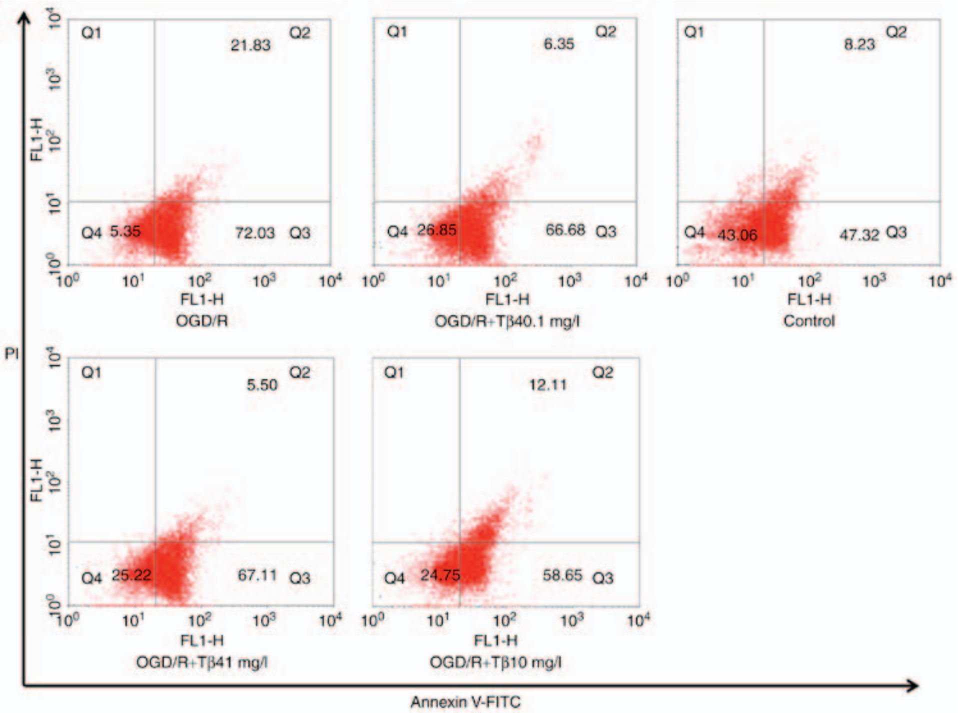

Figure 4. Influence of Tβ4 on OGD/R-induced cell apoptosis determined by flow cytometry. Quantitative analysis of Annexin V-FITC/PI staining in PC12 cells under OGD/R conditions and pre-treatment with different doses of Tβ4 by flow cytometry. The results indicated that pre-treatment with different concentrations of Tβ4 (0.1, 1 or 10 mg/l) significantly increased the percentage of surviving cells (Q1) after exposure to OGD/R (26.85±0.61, 25.22±0.53 and 24.75±0.50%, respectively, vs. 5.35±0.13% in the OGD/R group; n=3) and reduced the percentage of cells in late apoptosis/necrosis (Q2; 6.35±0.34, 5.50±0.23 and 12.11±1.35%, respectively, vs. 21.83±0.57% in the OGD/R group). In addition, the high concentration of Tβ4 reduced the number of early apoptotic cells (Q3; 58.65±0.94 vs. 72.03±1.32% in the OGD/R group). Tβ4, thymosin β4; OGD/R, oxygen-glucose deprivation and reoxygenation; FITC, fluorescein isothiocyanate; PI, propidium iodide; Q, quadrant.

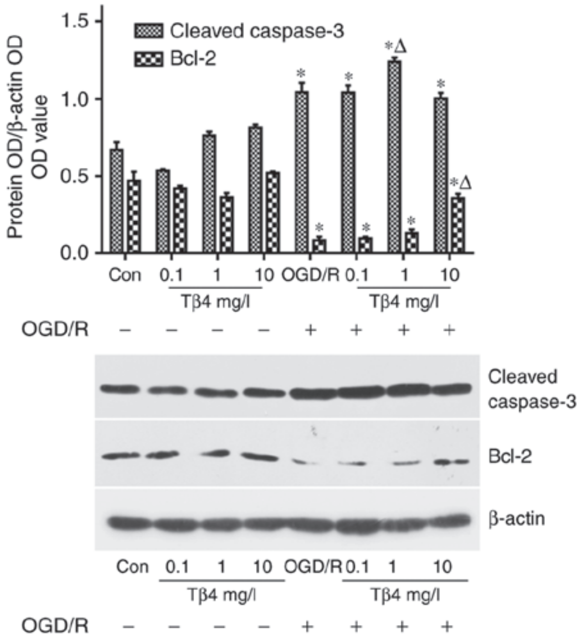

Figure 5. Influence of Tβ4 on the expression of apoptosis-associated proteins. Pre-treatment with Tβ4 led to an upregulation in the expression of the anti-apoptotic protein Bcl-2 in PC12 cells under OGD/R conditions. Values are expressed as the mean ± standard deviation. *P<0.05 vs. the control group; $^{\Delta}$P<0.05 vs. the OGD/R group. Bcl-2, B-cell lymphoma 2; Tβ4, thymosin β4; OGD/R, oxygen-glucose deprivation and reoxygenation; OD, optical density.

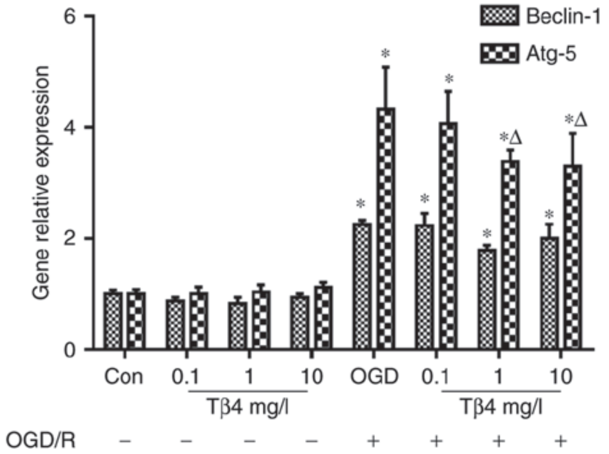

Figure 6. Influence of Tβ4 on the expression of autophagy-associated genes. Polymerase chain reaction analysis indicated that OGD/R upregulated the expression of Beclin-1 and Atg-5 relative to the control treatment; however, pre-treatment with 1 or 10 mg/l Tβ4 led to a downregulation of the expression of Beclin-1 and Atg-5 in PC12 cells under OGD/R conditions. *P<0.05 vs. the control group; $^{\Delta}$P<0.05 vs. the OGD/R group. Tβ4, thymosin β4; OGD/R, oxygen-glucose deprivation and reoxygenation; Atg-5, autophagy-related protein-5.

hypoxia-ischemia-induced autophagy and protected against neuronal death after cerebral ischemia. Koike *et al* (19) also demonstrated that inhibition of autophagy prevents neuronal death after hypoxic-ischemic injury, suggesting that autophagy is involved in cerebral ischemia.

Ischemia/reperfusion is known to stimulate autophagy through a Beclin-1-dependent mechanism (28). Furthermore, P62, a long-lived protein, which is assembled on selective

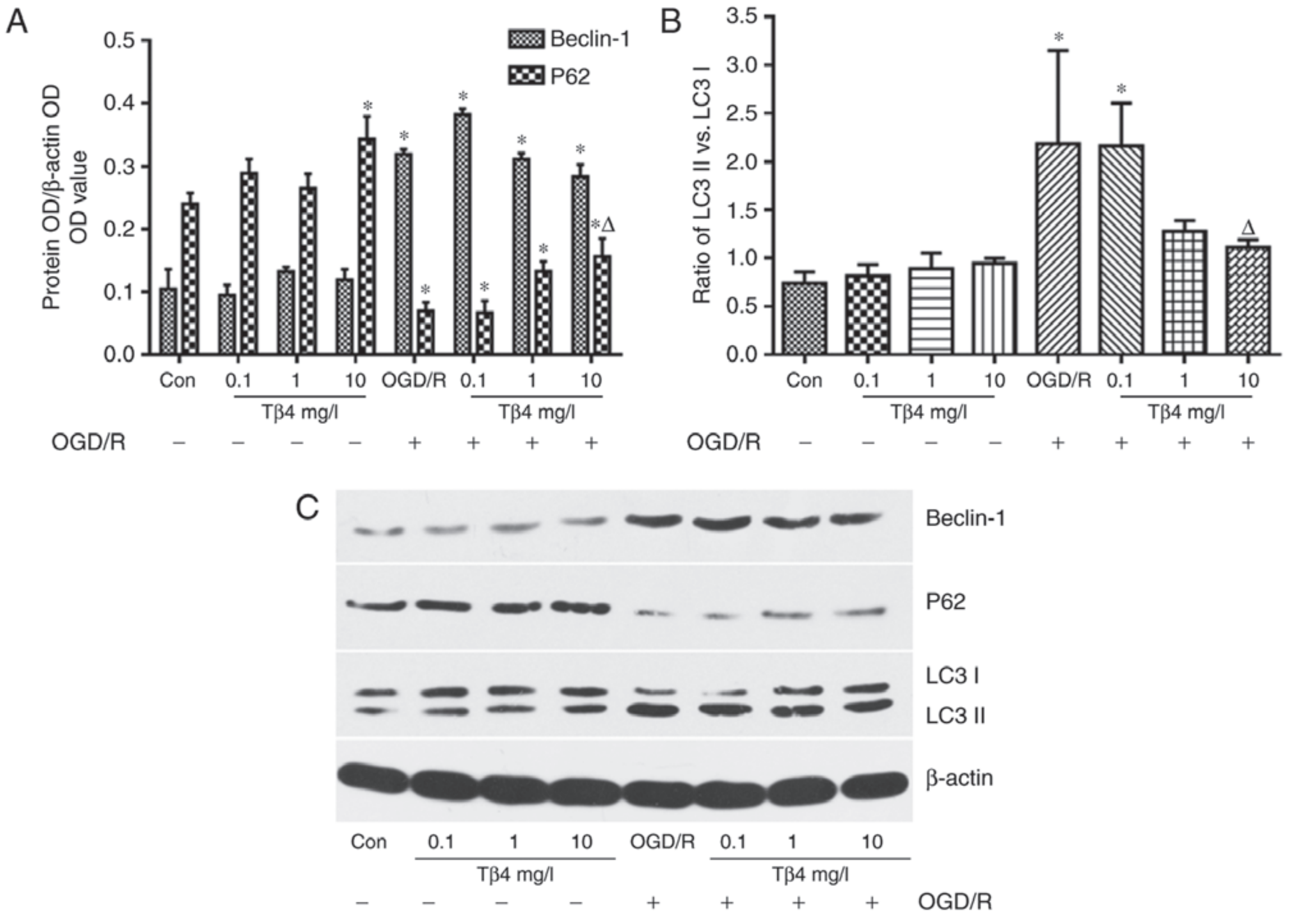

Figure 7. Influence of Tβ4 on the expression of autophagy-associated proteins. (A) The results indicated that pre-treatment with Tβ4 caused an upregulation of P62, and (B) a downregulation of the LC3II vs. LC3 I ratio in PC12 cells under OGD/R conditions. (C) Western blot analysis results. *P<0.05 vs. the control group; $^{\Delta}$P<0.05 vs. the OGD/R group. Tβ4, thymosin β4; OGD/R, oxygen-glucose deprivation and reoxygenation; LC3, microtubule-associated protein 1 light chain 3; OD, optical density.

autophagic cargos and preferentially degraded via autophagy, was assessed in the present study. P62 has been reported to be a possible marker of autophagic flux *in vivo* (29) and it also regulates autophagy (30).

In the present study, it was demonstrated that OGD/R increased the mRNA and protein expression of Beclin-1 and increased Atg-5 mRNA expression, indicating that OGD/R enhanced autophagy in PC12 cells. By contrast, P62 expression was significantly reduced after OGD/R. Of note, pre-treatment with 1 or 10 mg/l Tβ4 led to a downregulation of the mRNA expression of Atg-5, as well as the ratio of LC3 II vs. LC3 I, and an upregulation of the protein expression of P62.

Autophagy is closely associated with apoptosis (31). As mentioned above, autophagy during OGD/R is mediated by a Beclin-1-dependent pathway. Beclin-1, an autophagy-associated protein that contains a Bcl-2 homology-3 (BH3) domain (32), may be inhibited via activation of apoptosis-associated proteins that possess BH3-binding domains, including Bcl-2 (33). Therefore, Bcl-2 may reduce the pro-autophagic activity of Beclin-1, while upregulation of Bcl-2 may decrease autophagic cell death and reduce cellular autophagy by binding to Beclin-1.

Based on the finding that Tβ4 increased the expression of Bcl-2, it was hypothesized that the anti-autophagic effect of Tβ4 may be linked to the increased Bcl-2 expression, which may have promoted the interaction of Bcl-2 with Beclin-1. Tβ4 also increased the expression of the autophagy regulatory protein P62, thereby inhibiting autophagy, but the specific mechanisms require to be elucidated in further studies.

In conclusion, the present study demonstrated that OGD/R in PC12 cells induced excessive autophagic flux, likely leading to autophagic cell death. Tβ4 was demonstrated to reduce oxidative stress-induced cell damage and inhibit cell apoptosis and autophagy to partly prevent OGD/R-induced injury. The ability of Tβ4 to protect against OGD/R in PC12 cells may provide new opportunities for clinical therapeutic strategies in the future.

Competing interests

The authors declare that they have no competing interests.

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