Neuroscience Letters 556 (2013) 20–25

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

N-benzylcinnamide protects rat cultured cortical neurons from β-amyloid peptide-induced neurotoxicity

Wipawan Thangnipon[a,*], Nicha Puangmalai[a], Vorapin Chinchalongporn[a], Chanati Jantrachotechatchawan[a], Narisorn Kitiyanant[a,b], Rungtip Soi-ampornkul[c], Patoomratana Tuchinda[d], Saksit Nobsathian[e]

[a] *Research Center for Neuroscience, Institute of Molecular Biosciences, Mahidol University, Salaya, Nakhonpathom, Thailand*
[b] *Stem Cell Research Group, Institute of Molecular Biosciences, Mahidol University, Salaya, Nakhonpathom, Thailand*
[c] *Department of Biochemistry, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand*
[d] *Department of Chemistry, Faculty of Science, Mahidol University, Bangkok, Thailand*
[e] *Nakhonsawan Campus, Mahidol University, Amphoe Phayuhakiri, Nakhonsawan, Thailand*

HIGHLIGHTS

- Treatment with PT-3 alleviated $A\beta_{1-42}$-induced cell death and morphological changes.
- PT-3 also reduced production of ROS induced by $A\beta_{1-42}$.
- PT-3 prevents $A\beta_{1-42}$-induced toxicity by reducing oxidative stress and inflammation.
- PT-3 effects may involve inhibition of JNK and p38, but not ERK1/2 and Akt.

ARTICLE INFO

Article history:
Received 25 June 2013
Received in revised form 4 September 2013
Accepted 29 September 2013

Keywords:
Alzheimer's disease
Amyloid β-peptide
N-benzylcinnamide
Antioxidant
Apoptosis
Anti-inflammation
Mechanism

ABSTRACT

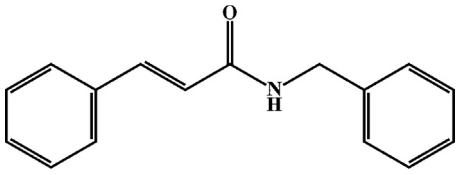
The pathogenesis of Alzheimer's disease involves an amyloid β-peptide (Aβ)-induced cascade of elevated oxidative damage and inflammation. The present study investigates the protective effects and the underlying mechanisms of *N*-benzylcinnamide (PT-3), purified from *Piper submultinerve*. Against Aβ-induced oxidative stress and inflammation in rat primary cortical cell cultures. Pre-treatment with 10–00 nM PT-3 significantly attenuated neuronal cell death induced by 10 μM $A\beta_{1-42}$. PT-3 was found to enhance cell viability through a significant reduction in the level of reactive oxygen species, down-regulated expression of pro-apoptotic activated caspase-3 and Bax, increased expression of anti-apoptotic Bcl-2, and mitigation of Aβ-induced morphological alterations. Regarding its effects on inflammatory responses, PT-3 pre-treatment decreased the expression of pro-inflammatory cytokines IL-1β and IL-6. The mechanisms of PT-3 neuronal protection against inflammation may be associated with the mitogen-activated protein kinases (MAPK) pathway. $A\beta_{1-42}$-induced phosphorylation of JNK and p38 MAPK was inhibited by pretreatment with PT-3 in a dose-dependent manner. However, phosphorylation of ERK1/2 was not affected by either PT-3 or $A\beta_{1-42}$. PT-3 did not stimulate Akt phosphorylation, which was inhibited by $A\beta_{1-42}$. These findings suggest that PT-3 protects neurons from $A\beta_{1-42}$-induced neurotoxicity through its anti-apoptotic, anti-oxidative, and anti-inflammatory properties with inhibition of JNK and p38 MAPK phosphorylation as the potential underlying mechanism.

1. Introduction

Alzheimer's disease (AD) is characterized by progressive loss of memory and impairment of higher cognitive functions. Pathological hallmarks of human AD brains are extracellular senile plaques composed of amyloid-β (Aβ) and intracellular neurofibrillary tangles [22]. $A\beta_{1-42}$ is the form that prominently increases in the brains of AD patients [7]. The mechanisms of Aβ-induced cytotoxicity have not been completely elucidated. Protection of neurons from Aβ-induced toxicity with antioxidants such as resveratrol [13] and the anti-inflammatory compound C16 (double-stranded RNA dependent protein kinase inhibitor) [4] demonstrate the importance of these pathways in the effects of Aβ. Natural plant-derived chemicals have advantages over synthetic compounds since they generally have lower toxicity and fewer side effects,

* Corresponding author. Tel.: +66 24419003x1203; fax: +66 24419003x1311.
E-mail addresses: wipawan.tha@mahidol.ac.th, grwtn@mahidol.ac.th (W. Thangnipon).

0304-3940/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neulet.2013.09.071



Chemical structure of *N*-benzylcinnamide

Fig. 1. Chemical structure of *N*-benzylcinnamide.

as demonstrated in experiments with resveratrol and *N*-trans-feruloyltyramine (NTF) [26].

N-benzylcinnamide (PT-3) (Fig. 1), purified from *Piper submultinerve* [21], has been suggested to have the capacity to protect neurons from Aβ-induced neurotoxicity. In our previous study, we discovered that NTF attenuates Aβ-induced cell death by inhibiting the generation of reactive oxygen species (ROS) and reducing the expression of pro-apoptotic proteins such as activated caspase-3 and Bax [26]. PT-3 shares several common structural characteristics with NTF, such as a large conjugated Pi-system and an amide group, which may serve as effective radical scavenging moieties [3]. In the present study, we examined the mechanisms of PT-3 neuroprotection against the neuronal insult of $A\beta_{1-42}$ treatment.

2. Methods

Enriched primary neuronal cell cultures were prepared from cerebral cortices of 10–12 Wistar rat fetuses at embryonic day-17 according to the previously described procedure [26]. On day 5, cultures were treated with 1, 2.5, 5, 10, 25, 50 and 100 nM PT-3 for 1 h prior to incubation with 10 μM oligomeric $A\beta_{1-42}$ (Keck Biotechnology, USA), a sublethal dose [6], for 24 h. Cultures were processed for Western Blot analysis and cell viability evaluation immediately after the incubation.

Morphological changes of neurons were visualized with phase-contrast microscopy. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [9]. Cultures were incubated with 0.5 mg/ml MTT for 3 h at 37 °C at which point the supernatant was discarded. The remaining blue formazan crystals were dissolved in DMSO and absorbance was measured at 570 nM using a microplate reader.

Intracellular ROS levels were evaluated using 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) [16]. Cells (5×10^5 cells/ml) were seeded in 96-well plates and cultured for 5 days. Cells were incubated with 50 μM DCFH-DA at 37 °C for 45 min in the dark before treatment with 2.5, 5, 10 and 25 nM PT-3 for 1 h. The cells were then exposed to 10 μM $A\beta_{1-42}$ for 24 h and DCF fluorescence was quantified using a Multi-Detection microplate reader with excitation and emission wavelength of 485 and 530 nm, respectively.

Following treatment with PT-3, neurons were washed with PBS and lyzed with lysis buffer. Lysates were placed on iced for 30 min and then centrifuged at 14,000 × *g* for 5 min. Equal amounts of supernatant protein were separated using 10% sodium dodecyl sulfate-polyacrylamide gel-electrophoresis at 150 V for 70–90 min. Proteins were then transferred onto polyvinylidene fluoride membranes at 80 V for 2 h. Membranes were treated with 5% non-fat dried milk in 0.1% Tween-20 in Tris-buffered saline (TBS-T) at room temperature for 1 h. Membranes were probed with the following primary antibodies in TBS-T overnight at 4 °C at the specified dilution: 1:500 mouse anti-Bcl-2, -Bax, -IL-6; 1:1000 rabbit anti-IL-1β (Santa Cruz); 1:500 rabbit anti- activated caspase-3, -phospho-p38, -p38; 1:1000 rabbit anti-phospho-ERK1/2, -ERK1/2, -phospho-JNK, -JNK; 1:2000 rabbit anti-phospho-Akt, -Akt, or 1:2500 rabbit anti-actin antibodies (Cell Signaling). After washing with TBS-T, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3500, GE Healthcare) at room temperature for 1 h. Blots were detected using enhanced chemiluminescence on X-ray film and band intensity was quantified using densitometry (ImageJ program).

Statistical significance of differences was determined by one-way analysis of variance (ANOVA), followed by the SNK post-test. Results are considered statistically significant at $p < 0.05$. All data are presented as mean ± SEM of 3 independent experiments conducted in triplicate.

3. Results

Cells treated with 10 μM $A\beta_{1-42}$ displayed cell shrinkage and broken fibers compared to normal neuronal cells, as assessed by phase-contrast microscopy (Fig. 2A and B). Pre-treatment with 10 nM PT-3 significantly attenuated $A\beta_{1-42}$ – induced morphological abnormalities (Fig. 2C). In addition, pre-treatment of neuronal cell cultures with 10–100 nM PT-3 significantly reduced $A\beta_{1-42}$-induced neuronal cell death as assessed by MTT assay (Fig. 2D). PT-3 treatment at any concentration alone did not result in cytotoxicity. As expected, ROS production in 10 μM $A\beta_{1-42}$-treated neuronal cells is significantly increased compared to the control (Fig. 3). Pre-treatment of 2.5–25 nM PT-3 decreased ROS levels in $A\beta_{1-42}$-treated cultures. However, PT-3 treatment alone at any concentration did not significantly alter ROS generation compared to the control.

Apoptosis was confirmed as the mechanism of neuronal cell death following $A\beta_{1-42}$ by the elevated level of activated caspase-3 in cell treated with 10 μM $A\beta_{1-42}$ (Fig. 4A). PT3 treatment at 5–25 nM significantly lowered activated caspase-3 expression in a dose-dependent manner. The level of the pro-apoptotic protein Bax was significantly increased in $A\beta_{1-42}$-treated cultures compared to the control. Similarly, $A\beta_{1-42}$-induced Bax expression was significantly decreased by 25 nM PT-3 (Fig. 4B). While the level of the anti-apoptotic protein Bcl-2 was not significantly altered by 10 μM $A\beta_{1-42}$ compared to the control, pre-treatment with 25 nM PT-3 significantly increased Bcl-2 (Fig. 4C). Additionally, the expression of IL-1β was not significantly increased in 10 μM $A\beta_{1-42}$-treated cultures compared to the control, but 10 and 25 nM PT-3 significantly inhibited IL-1β production (Fig. 5A). In contrast, the level of IL-6 was significantly elevated by 10 μM $A\beta_{1-42}$ compared to the control and this increase in IL-6 was significantly reduced by 25 nM PT-3 (Fig. 5B). As shown in Fig. 6A and B, incubation of neuronal cell cultures with 10 μM $A\beta_{1-42}$ induced JNK and p38 phosphorylation, but not ERK1/2 phosphorylation (Fig. 6C). Pre-treatment with 2.5–25 nM PT-3 dramatically inhibited $A\beta_{1-42}$-induced JNK and p38 phosphorylation, but had no effect on ERK1/2 phosphorylation. Akt phosphorylation was inhibited by 10 μM $A\beta_{1-42}$, but not altered by PT-3 treatment (Fig. 6D).

4. Discussion

In the present study we have demonstrated that in cultured cortical neurons exposure to 10 μM $A\beta_{1-42}$ for 24 h could lead to apoptotic cell death, as manifested by morphological changes and the expression of apoptotic proteins, including activated caspase-3 and pro-apoptotic Bax. Elevated levels of ROS, which are associated with apoptotic cell death, were also increased by treatment with $A\beta_{1-42}$. These effects could be significantly attenuated by pre-treatment with 2.5–25 nM PT-3 for 1 h. PT-3 decreased ROS levels in a non-dose dependent manner. Treatment with PT-3 at any concentration yielded the similar results, but the biggest decrease was found at 2.5 nM PT-3. According to LaFerla et al. [14], ROS generation-induced by $A\beta_{1-42}$ treatment is not only