Ciliary neurotrophic factor protects SH-SY5Y neuroblastoma cells against $\text{A}\beta_{1-42}$-induced neurotoxicity via activating the JAK2/STAT3 axis

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Abstract

The neurotoxicity of aggregated amyloid beta (Aβ) has been implicated as a critical cause in the pathogenesis of Alzheimer’s disease (AD), which leads to neuronal cell damage by inducing oxidative stress and consequently triggering cell apoptosis. Recently, Aβ-dependent inactivation of the Janus tyrosine kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signaling pathway was found to play a critical role in the memory impairment related to AD. Previous research indicated that JAK2/STAT3 axis inactivation might be the result of aberrant reactive oxygen species (ROS) generation induced by Aβ in neurons. As the JAK2/STAT3 axis is a major transducer of ciliary neurotrophic factor (CNTF)-mediated neuroprotective activity, this study extensively evaluated whether activation of the JAK2/STAT3 axis by CNTF was responsible for the neuroprotective effect of this protein against $\text{A}\beta_{1-42}$-induced cytotoxicity, oxidative injury and cell apoptosis in human SH-SY5Y neuroblastoma cells. Our data showed that CNTF could attenuate or restore cell injury induced by $\text{A}\beta_{1-42}$ in human SH-SY5Y neuroblastoma cells through activating the JAK2/STAT3 signaling pathway. Furthermore, CNTF strikingly prevented $\text{A}\beta_{1-42}$-induced mitochondrial dysfunction and activation of mitogen-activated protein kinases (MAPKs), an effect that could be potently attenuated by the specific JAK2 inhibitor AG490. In summary, this study confirmed the detailed mechanism accounting for CNTF’s protective effect against $\text{A}\beta_{1-42}$-induced cytotoxic events in human SH-SY5Y neuroblastoma cells – information which might significantly contribute to better understanding of the mechanism of action of CNTF as well as providing a novel target in AD therapy.

Key words: Alzheimer’s disease, $\text{A}\beta_{1-42}$, ciliary neurotrophic factor, JAK2/STAT3.

Introduction

Alzheimer’s disease (AD) is the leading cause of dementia in aging adults, which currently has about 36 million cases worldwide [1,3,14]. Alzheimer’s disease causes a large loss in brain weight and volume and affects some brain regions and neuronal populations [24,36]. Although the pathogenesis of AD...
remains unclear, the key event appears to be the formation of a peptide known as amyloid beta (Aβ), with two major forms, Aβ1-40 and Aβ1-42. Amyloid β clusters into amyloid plaques on the blood vessels and the outer surface of neurons of the brain, leading to the killing of neurons [10]. The mechanism of Aβ-induced neurotoxicity is inconclusive. New evidence suggests that Aβ-dependent inactivation of the Janus tyrosine kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) axis in hippocampal neurons causes cholinergic dysfunction via pre- and post-synaptic mechanisms, which leads to memory impairment related to AD [7,8]. However, intracellular events accounting for the mechanism of this action in neurons remain elusive.

Oxidative stress is the redox state resulting from an imbalance between the generation and detoxification of reactive oxygen species (ROS) [33]. Increased cellular oxidative stress has been implicated in the pathogenesis of several neurodegenerative diseases, including Alzheimer’s disease [27]. Aβ peptides are key stimuli of ROS generation, which could infuse into the brain through microdialysis probes and increase ROS production in an NMDA receptor- and nitric oxide-dependent manner [4]. A previous study indicated that aberrant activation of ROS generation might block activation of the JAK2/STAT3 axis in neurons, consequently disrupting the effect of neurotrophic factor and inducing neuronal damage in neurodegenerative diseases [19]. Thus, activation of the JAK2/STAT3 axis might induce a protective effect against oxidative stress induced by Aβ.

The major role of neurotrophic factors (NTFs) in synapse function along with evidence that synaptic failure is a critical early event in AD has put NTFs at the forefront of neuroprotective strategies for AD [32]. Ciliary neurotrophic factor (CNTF), a classic member of the NTFs, was first described as a growth factor that supports survival of chick ciliary ganglion neurons. Later, it was shown to be an important factor in the central and peripheral neurons of the nervous system [30]. CNTF binds to its α-receptor and two signal-transducing transmembrane subunits, LIFRβ and gp130, specifically activating the JAK2/STAT3 signaling pathway and thus preventing neuron death and facilitating axonal regeneration after nerve injury [4,28]. The neuroprotective effect of CNTF in AD has been previously reported; it might be mediated through modulating brain plasticity by promoting neurogenesis [12]. However, the precise cellular and molecular mechanisms underpinning such a neuroprotective effect remain far from clear.

In this study, we aimed to elucidate the role of the JAK2/STAT3 axis during the neuroprotective effect of CNTF against Aβ1-42-induced oxidative injury in human SH-SYSY neuroblastoma cells, which has been widely used as a typical model for AD [17,38]. Furthermore, we also elucidated the molecular events of both the upstream and downstream signaling pathways involved in this cellular process.

Material and methods

Materials and chemicals

Recombinant human CNTF was produced in Escherichia coli by our laboratory [37]. All cell culture reagents were purchased from Gibco (NY, USA). Aβ1-42, AG490, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (MO, USA). Antibodies against Bcl-2, Bcl-xL and β-actin were obtained from Santa Cruz Biotechnology (CA, USA). Antibodies against JAK2, STAT3, phospho-JAK2, phospho-STAT3, JNK, ERK, p38, phospho-JNK, phospho-ERK and phospho-p38 were purchased from Abcam (MA, USA). Caspase-3 and caspase-9 fluorometric assay kits were obtained from BioVision (SF, USA). All other chemicals and reagents were of analytical grade.

Cell culture and treatment

The human neuroblastoma cell line (SH-SYSY) was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). SH-SYSY cells were cultured in flasks at 37°C under an atmosphere of 5% CO2/95% air in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Before the experiments, Aβ1-42 peptide was diluted to the desired concentrations and maintained for 3 days at 37°C for oligomerization. For the experiments, the cells were detached, re-seeded on plates and then incubated with or without drugs for the indicated time.
Cell growth assay

Cell proliferation was measured by the MTT assay as described before [22,43]. Briefly, 10 μl of MTT stock solution (5 mg/ml) was added to each well and incubated for 4 h at 37°C. The culture medium was then removed and 100 μl of DMSO was added to dissolve the formazan crystals. After mixing, absorbance was measured at 570 nm with an ELISA reader (Bio-Rad, CA, USA). Cell viability was also analyzed by the trypan blue exclusion assay. After treatment with the indicated drugs, cells were washed with phosphate buffer saline (PBS) twice at the end of the incubation period and trypsinized. The cells were re-suspended and subjected to trypan blue staining and then cell counting. Cell viability was expressed as a percentage value in relation to that of the control.

Cell apoptosis assay

Apoptosis of cells was examined by double staining with Annexin V-FITC and PI. After treatment, cells were washed twice with ice-cold PBS and re-suspended in 300 μl of binding buffer (Annexin V-FITC kit, Becton-Dickinson, CA, USA) containing 10 μl of Annexin V-FITC stock and 10 μl of PI. After 15 min incubation at room temperature in the dark, the samples were analyzed by flow cytometry. The Annexin V+/PI- cells were considered as apoptotic cells, the percentage of which was calculated by CellQuest software (Becton-Dickinson, CA, USA).

Reactive oxygen species detection

Reactive oxygen species (ROS) production was measured by flow cytometry using DCFH-DA staining. DCFH-DA is cleaved intracellularly by nonspecific esterases and transforms to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. Briefly, after treatment, the cells were incubated with DCFH-DA (20 μM) for 30 min at 37°C in the dark. After washing twice with PBS, the fluorescence intensity was measured by the microplate reader (Molecular Devices, CA, USA) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The level of ROS was expressed as a percentage value in relation to the control.

Western blot analysis

The treated cells were collected and lysed in ice-cold RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM ethylene diamine tetraacetic acid (EDTA), 20 mM NaF, 100 mM Na2VO4, 1% Nonidet P-40 (NP-40), 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/ml aprotinin and 10 mg/ml leupeptin) for 30 min. After centrifugation, protein concentration was determined by the Bradford method [21]. Cell lysates were separated by electrophoresis on 15% SDS-polyacrylamide gel (SDS-PAGE) and transferred onto PVDF membrane. After blocking with 5% bovine serum albumin (BSA) in the mixture of Tris-buffered saline and Tween-20 (TBST) for 1 h, membranes were incubated with primary antibody (diluted in 1 : 500) overnight and followed by incubation with secondary antibody (diluted in 1 : 1000) for 1 h at room temperature. Protein bands were visualized using the ECL assay kit (Beyotime, Nantong, China). The density of each band was normalized to the expression of β-actin.

Caspase activity assay

Caspase activity was assessed by fluorometric assay [38]. Cells were collected and lysed in caspase assay buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA, 10 mM digitonin and 2 mM dithiothreitol (DTT). Protein was separated by centrifugation, the concentration of which was determined as previously described [21]. An equal amount of protein from each sample was incubated with caspase-3 substrate DEVD-AFC or caspase-9 substrate LEHD-AFC for 30 min at 37°C. The caspase activity was assessed by a spectrofluorometer (Molecular Devices, CA, USA) with an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

Statistical analysis

Biostatistical analyses were conducted with the Prism 5.0 and SPSS 16.0 software packages. Data were represented as mean ± SEM. Comparisons between experimental and control groups were performed by one-way ANOVA and differences were considered to be statistically significant when p value was less than 0.05.

Results

CNTF induces JAK2/STAT3 activation in Aβ1-42-treated SH-SY5Y cells

It is known that CNTF prevents neuronal cell death and facilitates axonal regeneration after nerve injury.
CNTF protects SH-SYSY cells via JAK/STAT3 axis

CNTF protects SH-SYSY cells from Aβ1-42-induced cytotoxicity

Aβ1-42 impairing neuronal cells has been considered as one of the major causes of AD [31]; therefore, in this study, Aβ1-42 was employed as a neurotoxicant. The neuroprotective effect of CNTF against Aβ1-42-induced cytotoxicity was evaluated as the viability of SH-SYSY cells using the MTT assay and trypan blue exclusion assay. As shown in Fig. 2, Aβ1-42 (20 μM) exhibited a remarkable inhibitory effect on the growth of SH-SYSY cells. However, this cytotoxic effect was attenuated by co-treatment with CNTF (10 ng/ml). To investigate the involvement of JAK2/STAT3 signaling in the protective effect of CNTF against Aβ1-42 induced cytotoxicity, we assessed cell viability with the co-treatment of the JAK2 inhibitor AG490 in the presence of both CNTF and Aβ1-42. Our results indicated that the suppressive effect of CNTF on Aβ1-42-induced cytotoxicity was significantly diminished in the presence of AG490 in human SH-SYSY neuroblastoma cells.
CNTF protects SH-SY5Y cells from Aβ1-42-induced oxidative injury

Aβ1-42-induced oxidative stress was considered as crucial to the pathophysiology of AD [6]. In this study, oxidative stress was assessed by measuring the intracellular ROS level using the ROS-sensitive fluorescence probe DCF. As shown in Fig. 3, after exposure to Aβ1-42 (20 μM) for 6 h, the intracellular ROS level was significantly increased (198.5% of the control), which suggested induced oxidative stress in SH-SY5Y cells. After co-treatment with CNTF (10 ng/ml), the intracellular ROS level was significantly decreased compared to that of Aβ1-42 treatment alone (122.3% of the control). In addition, AG490 inhibited the protective effect of CNTF on Aβ1-42-induced oxidative stress in SH-SY5Y cells. Our results suggested that CNTF could prevent Aβ1-42-induced oxidative injury in human SH-SY5Y neuroblastoma cells, possibly via activating the JAK2/STAT3 signaling pathway.

CNTF inhibits Aβ1-42-induced mitochondrial dysfunction

Mitochondrial dysfunction is an early event of cell apoptosis [39]. Increasing evidence indicates that Aβ induces oxidative injury and neuronal apoptosis through mediating mitochondrial dysfunction [25]. To investigate the effect of CNTF on Aβ1-42-induced mitochondrial dysfunction, the expression of anti-apoptotic members (Bcl-xL and Bcl-2) and activity of caspases were assessed, these genes being downstream targets of the JAK2/STAT3 pathway. As shown in Fig. 5, Aβ1-42 (20 μM) treatment significantly down-regulated the expression of Bcl-xL and Bcl-2 and up-regulated the activity of initiator caspase-9 and effector caspase-3. However, CNTF (10 ng/ml) co-treatment substantially reversed the regulatory effect of Aβ1-42. In addition, AG490 attenuated the protective effect of CNTF on Aβ1-42-induced mitochondrial dysfunction in human SH-SY5Y neuroblastoma cells.

CNTF inhibits Aβ1-42-induced JNK and ERK activation

Accumulating evidence has suggested that the MAPK signaling pathway plays an important role in neuronal death in AD [42]. Aβ1-42-induced oxidative stress influences the decision of susceptible neurons to undergo either apoptosis or proliferation, a process which is likely mediated through the MAPK signaling pathway [34]. In this study, the phosphorylation of JNK, ERK and p38, the most extensively studied vertebrate MAPKs, was assessed by western blot analysis. As shown in Fig. 6, Aβ1-42 (20 μM) treatment significantly induced the phosphorylation of JNK and ERK but not p38 (data not shown), an effect that was inhibited by CNTF (10 ng/ml). Again, the...
Fig. 4. The effect of CNTF on AP1-42-induced apoptosis in SH-SYSY cells. Cells were treated with AP1-42 (20 μM) in the absence or presence of CNTF (10 ng/ml) and AG490 (10 μM) for 24 h. Cell apoptosis was evaluated by flow cytometry. (A) Flow cytometry analysis of cell apoptosis using Annexin V-FITC/PI dual staining. (B) Percentage distribution of apoptotic cells. Results are shown as mean ± SEM of three experiments with each including triplicate sets. a: p < 0.05 vs. control, b: p < 0.05 vs. AP1-42, c: p < 0.05 vs. AP1-42 + CNTF.
effect of CNTF on Aβ₁₋₄₂-induced cytotoxicity in human SH-SY5Y neuroblastoma cells in the current study.

Furthermore, we elucidated the involvement of the critical signaling pathway in the molecular response of CNTF.

CNTF belongs to the IL-6 family of cytokines, signaling of which is mainly mediated through activation of the receptor-associated JAK2/STAT3 pathway [18]. Recent studies have demonstrated that the JAK2/STAT3 pathway is involved in multiple physiological processes of the nervous system [11, 41]. Chiba et al. reported that Aβ-dependent inactivation of the JAK2/STAT3 axis could lead to memory loss through cholinergic dysfunction [9]. In the current study, our results demonstrated that the JAK2/STAT3 pathway was inactivated upon the treatment with Aβ₁₋₄₂ in human SH-SY5Y neuroblastoma cells, whereas the further co-treatment with CNTF significantly stimulated the phosphorylation of JAK2 and STAT3 in a dose-dependent manner and protected cells against Aβ₁₋₄₂-induced cytotoxicity. Additionally, the JAK2 inhibitor AG490 largely attenuated the protective effect of CNTF on Aβ₁₋₄₂-induced toxicity in human SH-SY5Y neuroblastoma cells. Our study

Discussion

Alzheimer’s disease is the most common neurodegenerative dementia in the elderly, affecting cognition, behavior and functioning due to neuron loss [35]. Neurodegeneration possibly results from the abnormal accumulation of extracellular Aβ; therefore, Aβ is a promising therapeutic target of AD [20]. CNTF is a pleiotropic cytokine with neurotrophic properties for a number of neurons in vitro and in vivo. It is also one of the most active neurotrophic factors widely studied in promoting neurogenesis [15, 23]. Previous studies reported that treatment of CNTF in two AD mice models prevented Aβ oligomer-induced neuronal damage and neurobehavioral impairments [29]; however, the molecular mechanism underpinning this effect was largely unknown. Consistently, we confirmed the protective effect of CNTF on Aβ₁₋₄₂-induced cytotoxicity in human SH-SY5Y neuroblastoma cells in the current study.

Fig. 5. The effect of CNTF on Aβ₁₋₄₂-induced mitochondrial dysfunction in SH-SY5Y cells. Cells were treated with Aβ₁₋₄₂ (20 μM) in the absence or presence of CNTF (10 ng/ml) and AG490 (10 μM) for 24 h. (A) Expression of Bcl-xL and Bcl-2 was assessed by western blotting. (B) Activity of caspase-3 and -9 was determined by fluorometric assay. Results are shown as mean ± SEM of three experiments with each including triplicate sets. a: p < 0.05 vs. control, b: p < 0.05 vs. Aβ₁₋₄₂, c: p < 0.05 vs. Aβ₁₋₄₂ + CNTF.

Fig. 6. The effect of CNTF on Aβ₁₋₄₂-induced JNK and ERK activation in SH-SY5Y cells. Cells were treated with Aβ₁₋₄₂ (20 μM) in the absence or presence of CNTF (10 ng/ml) and AG490 (10 μM) for 6 h. Expression of JNK and ERK was assessed by western blotting.
suggested that the JAK2/STAT3 pathway might be the major transducer of CNTF-mediated neuroprotective activity.

Oxidative stress is one of the early events in AD, and is also implicated as an important mediator of the onset, progression and pathogenesis of AD. Several studies have suggested that oxidative stress plays a key role in Aβ-mediated neuronal cytotoxicity by triggering or facilitating neurodegeneration through a wide range of molecular events that eventually lead to neuronal cell loss [6]. In our study, after exposure of SH-SYSY cells to Aβ1-42 for 6 h, the intracellular ROS level was significantly increased; however, co-treatment with CNTF potently inhibited this effect, suggesting that CNTF could prevent Aβ1-42-induced oxidative injury in SH-SYSY cells. Recent studies demonstrated that the JAK2/STAT3 pathway was involved in regulating oxidative stress in certain types of neurons. We also confirmed that activation of the JAK2/STAT3 pathway was involved in the protective effect of CNTF against Aβ1-42-induced oxidative injury.

To further investigate the molecular mechanisms underlying this neuroprotective effect, the ROS-related downstream signaling pathways including the mitochondrial pathway and MAPK cascades were monitored. Zhao et al. showed that the JAK2/STAT3 pathway could modulate the expression of anti-apoptotic members (Bcl-xL and Bcl-2) and activity of caspases [40]. Our present study also proved that activation of the JAK2/STAT3 pathway could reverse the decreased expression of Bcl-xL and Bcl-2 as well as activation of caspases induced by Aβ1-42 in human SH-SYSY neuroblastoma cells. Oxidative stress is one of the major stimuli of MAPK cascades, pathways widely involved in apoptotic signal transduction. Ghribi et al. reported that administration of Aβ1-42 into rabbit brain induced apoptosis accompanied with activation of JNK and ERK, but not p38 [13]. Our study showed that treatment with Aβ1-42 significantly induced the phos-

![Fig. 7. The proposed model of CNTF neuroprotective effect against Aβ1-42-induced cytotoxicity in human SH-SYSY neuroblastoma cells.](image-url)
phorylation of JNK and ERK in human SH-SY5Y neuroblastoma cells, an effect that could be abolished by co-treatment with CNTF. JNK and ERK pathways are also targets of the JAK2/STAT3 axis, and co-treatment with AG490 inhibited the effect of CNTF on Aβ_{1-42}-induced JNK and ERK activation. Our data suggested that CNTF could inhibit Aβ_{1-42}-induced mitochondrial dysfunction and MAPK activation via activating the JAK2/STAT3 pathway in human SH-SY5Y neuroblastoma cells.

In conclusion, our study extensively evaluated the protective effect of CNTF against Aβ_{1-42}-induced neurotoxicity in human SH-SY5Y neuroblastoma cells. More importantly, we provided evidence that such a neuroprotective effect of CNTF was largely mediated through activation of the JAK2/STAT3 signaling pathway (Fig. 7). Our findings might significantly contribute to better understanding of the mechanism of action of CNTF and constitute a basis for future development of neuronal growth factors as potential drugs for the treatment of AD.

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Disclosure

Authors report no conflict of interest.

References

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