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Overexpression of conserved dopamine neurotrophic factor (CDNF) in astrocytes alleviates endoplasmic reticulum stress-induced cell damage and inflammatory cytokine secretion

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ABSTRACT

Astrocyte damage and the disorders of cytokine secretion induced by endoplasmic reticulum stress (ERS) are crucial pathological processes in ischemic injury of the central nervous system (CNS), (e.g., ischemic reperfusion injury of the brain and spinal cord). ERS stimulates damage to astrocytes and the release of pro-inflammatory cytokines, which deteriorates CNS injury. This current study investigates whether the overexpression of conserved dopamine neurotrophic factor (CDNF) alleviates ER stress-induced cell damage and inflammatory cytokine secretion. We found that primary astrocytes showed both a successful transduction and a significant overexpression of CDNF protein following lentivirus application. Our results show that the percentage of LDH released as a result of ER stress was significantly lower in astrocytes with an overexpression of CDNF than in the control groups without CDNF overexpression, indicating that CDNF alleviates ER stress-induced astrocyte damage. The secretion and mRNA expression levels of pro-inflammatory cytokines were increased by tunicamycin, and this stimulation was significantly suppressed by an overexpression of CDNF, demonstrating that CDNF plays an important role in astrocyte inflammation and functioning by resisting ER stress. These findings suggest that primary astrocytes can be efficiently transduced with CDNF lentiviral vectors and that the overexpression of CDNF in astrocytes shows the potential to alleviate cell damage and proinflammatory cytokine secretion, which may represent a promising strategy for neuroprotection in the CNS.

1. Introduction

Astrocytes play an important role both in physiological and pathological process in the central nervous system (CNS) [1,2]. They respond swiftly to subtle changes in the microenvironment and secrete an array of pro-inflammatory and anti-inflammatory cytokines, chemokines, and trophic factors to modify the ambient microenvironment [3,4]. Astrocyte-derived factors are important in neuronal survival, neurogenesis and neuron repair [5,6]. Astrocyte damage and the disorders caused by cytokine secretion are crucial pathological processes in CNS ischemic injury (e.g., brain and spinal cord ischemic reperfusion injury).

In ischemic reperfusion injury, endoplasmic reticulum stress (ERS) is caused by hypoxic-ischemia, glucose starvation, ATP depletion, oxidative stress and Ca^{2+} homeostasis disorders. Astrocyte damage and apoptosis is then induced by ERS through the accumulation of unfolded or misfolded proteins in a subcellular organelle mainly referred to as a protein-folding factory known as the ER [7–10]. Tunicamycin, a naturally occurring antibiotic and a trigger of the ER stress response (ERSR), is used to imitate the condition of an astrocyte under ERSR, which will then cause cell damage and inflammatory responses *in vitro* [11].

Previous studies demonstrated that there is no effective therapy to treat CNS ischemic reperfusion injury; however, conserved dopamine neurotrophic factor (CDNF), a vertebrate-specific paralogue of the human mesencephalic-astrocyte-derived neurotrophic factor (MANF) [12], may provide a potential method. Previous evidence suggests that MANF is an ER stress response protein and is able to protect cells against ER stress-induced cell death *in vitro* [13]. However, the role of CDNF and the function of astrocytes in the ER stress response is not clear; therefore, we investigated the effect of overexpression of CDNF on ERS-induced cell damage and inflammatory cytokine secretion in astrocytes *in vitro*.

2. Materials and methods

2.1. Animals

Wistar rats were obtained from the Laboratory Animal Center of Shandong University. All animals were kept under controlled light/

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dark conditions (12/12 h), temperature (23 °C), and humidity (60%). In the handling and care of all animals, the International Guiding Principles for Animal Research were followed, as stipulated by the World Health Organization (1985) and as adopted by the Laboratory Animal Center of Shandong University. All efforts were made to minimize pain and the number of animals used.

2.2. Rat astrocyte isolation and culture

Primary astrocytes were prepared from neonatal Wistar rats post-natal day one, as previously described [14]. Briefly, the cortical tissues freed of meninges and blood vessels were mechanically dissociated, and the cell suspension was seeded at a density of 1×10^6 cells/ml in Dulbecco's modified Eagle medium (5.5 mmol/L glucose) (DMEM, Gibco, Grand Island, NJ, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NJ, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma–Aldrich, St. Louis, MO, USA). When the culture reached confluency, the microglia and oligodendrocytes were removed by an orbital shaker; the remaining cells, a majority of them astrocytes, were replated. Before each experiment, the plated cells were incubated with serum-free DMEM medium for 1 h.

2.3. Immunocytochemistry

Primary astrocytes were subcultured on sterile glass coverslips for 12–16 h, washed in PBS, fixed in 4% formaldehyde for 10 min at 37 °C, permeabilised in 0.2% Triton-X 100 for 10 min at room temperature, washed in PBS, and then blocked in 1% BSA in PBS for 20 min at room temperature. The cells were then incubated with primary antibodies (mouse monoclonal anti-GFAP 1:100, Abcam) overnight at 4 °C. Cells were then washed in PBS and incubated with secondary antibodies (Texas-Red-conjugated anti-mouse IgG 1:1000, Invitrogen) in PBS for 40 min at 37 °C. Images were captured using an EVOS fluorescence microscope (EVOS, AMG, WA, USA).

2.4. Recombinant CDNF lentiviral vector design and production

The lentivirus was produced from co-transfection with three plasmids (pMD2G, pSPAX, and the transfer plasmid vector plenti-his, kindly provided by Prof. Tang DQ of Shandong University). CDNF was amplified from a cDNA3.1-human CDNF plasmid constructed in our previous study [15] using the following primers: CDNF forward primer 5′-TAGGATCCATGTGGTGCGCGAGCCCAGT-3′, CDNF reverse primer 5′-TACTCGAGTCAGAGCTCTGTTTTGGGGT-3′. Recombinant lentiviral vectors plenti-CDNF were harvested and concentrated using the Lenti-X Concentrator (Clontech, TaKaRa, CA, USA) 72 h following co-transfection of the pMD2.G (3.4 μg), pSPAX (6.6 μg), and the transfer plasmid plenty-his (10 μg) into 293T cells cultured in DMEM (10% FBS). Transfections were performed using Lipofectamine (Invitrogen, Carlsbad, CA) with the manufacturer's recommendations. Lentiviral vectors plenti-his were also produced as a control group using the plasmid plenti-his instead of plenti-CDNF.

2.5. In vitro lentiviral vector transduction

Astrocytes were seeded in 6-well plates (Corning, Corning, NY) at a density of 5×10^4/well, and 1 ml Dulbecco's modified Eagle medium (5.5 mmol/L glucose) with 10% FBS was added to each well. Primary astrocytes were cultured for 24 h. Viral multiplicity of infection (MOI) was detected by Lenti-X GoStix (Clontech, TaKaRa, CA, USA). Primary astrocytes transduced with 5 MOI of the plenti-CDNF/plenti-his lentivirus were used in the following studies as the morphology of these cells was most similar to the non-transduced astrocytes. Following incubation at 37 °C in 5% CO_2 for 24 h, the virus-containing medium was removed and replaced with 1 ml of fresh culture medium per well. As plenti-CDNF does not contain a GFP-reporting gene, the transduction efficiency should be determined by measuring the CDNF protein using a Western blot.

2.6. Western blot analysis

Five days following lentiviral vector transduction, plenti-CDNF-expressing astrocytes were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested in a lysis buffer containing 1 mM PMSF. The extract was centrifuged at 12,000×*g* for 5 min at 4 °C to remove cell debris. Protein concentration was determined by a BCA protein assay kit according to the manufacturer's instructions. Equivalent amounts of protein (10 μg) for each sample were separated by 10% Acrylamide-SDS–PAGE using 5% stacking and 12% separating gels and were subsequently transferred to polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA). Primary antibodies (goat anti-CDNF, 1:1000 dilution, R&D Systems, Inc., Minneapolis, MN, USA) and a rabbit anti-goat immunoglobulin (IgG)-horseradish peroxidase (HRP) secondary antibody (1:30,000 dilution, Cell Signaling Technology, Danvers, MA) was applied. Equal amounts of protein-loading were confirmed by re-probing the membranes with the mouse anti-β-actin-HRP (1:10,000 dilution, Abcam). Immunoblots were visualized by chemiluminescence (Pierce Biotechnology, Rockford, IL) with exposure to autoradio-graph film (X-OMAT AR; Eastman Kodak, Rochester, NY).

2.7. Interventions of astrocytes after lentivirus transduction

Five days after the lentiviral vector transduction, the experimental plenti-CDNF-transfected astrocytes and the control plenti-his-transfected astrocytes were seeded in 6-well plates (Corning, Corning, NY) at a density of 5×10^4/well, and 1 ml Dulbecco's modified Eagle medium (5.5 mmol/L glucose) with 10% FBS was added to each well. After 24 h, the medium was removed and replaced with 1 ml of fresh culture medium without FBS and was allowed to sit in culture for the next 24 h. The cells were then treated with either 50 ng/ml of tunicamycin or a vehicle for the following studies [16]. Tunicamycin was dissolved in phosphate-buffered saline (PBS; pH 7.4), and the same volume of PBS was used as the vehicle.

2.8. LDH release assay

Five days after lentivirus transduction, plenti-CDNF and plenti-his-transfected astrocytes and non-transfected cells were treated with tunicamycin (50 ng/ml) for 5 h, which was expected to cause ER-stress. To measure the extent of damage to the cells, the stable cytosolic enzyme resulting from cell lysis, lactate dehydrogenase (LDH) was measured in the cell culture medium using an LDH-Cytotoxicity Assay Kit II (BioVision, CA, USA). Simply stated, the clear medium (100 μl/well) was transferred into an optically clear 96-well plate, then 100 μl of LDH Reaction Mix was added to each well, mixed and incubated at room temperature for 30 min. The absorbance at 450 nm was measured by a microplate reader spectrophotometer (Multiskan Ascent).

2.9. ELISA

After 5 h of the treatment of tunicamycin, cytokine (IL-1ß, IL-6, and TNF-α) levels in astrocyte culture medium were determined by ELISA, as described by the manufacturer (R&D Systems). ODs were determined using a Spectromax 190 microplate reader (Molecular Devices) at 450 nm. Cytokine concentrations in the

medium were determined from standards containing known concentrations of the proteins.

2.10. RNA extraction and real-time RT-PCR assay

Total RNA was isolated using a TRIzol reagent (Takara, Otsu, Shiga, Japan) from astrocytes treated with tunicamycin for 24 h. Identical amounts of RNA (0.5 ng) were reverse-transcribed into complement DNA (cDNA) by using a commercial RT-PCR kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. The primers and PCR conditions for amplification were shown in Table 1. The quantification data were analyzed with LightCycler analysis software version 4.0 (Roche Applied Science, Mannheim, Germany). The relative expression was normalized on the basis of β-actin. At least three independent experiments were conducted for each condition.

2.11. Statistical analysis

Quantitative data were presented as the mean ± SD of at least three independent experiments. Statistical analysis of data was performed using a Student's *t*-test or by one-way ANOVA using a Dunnett's test in multiple comparisons of means. Differences were considered to be statistically significant if the *P*-value was <0.05 (∗) or <0.01 (∗∗).

3. Results

3.1. Astrocyte culture and CDNF expression after lentivirus transfection

After three passages, astrocytes that appeared flat and polygonal were grown in a monolayer and detected by an EVOS fluorescence microscope. More than 95% of the cells were GFAP positive, which means that they were identified as astrocytes (Fig. 1A). CDNF expression in astrocytes after lentivirus transfection was measured by a Western blot. The expression of CDNF protein in astrocytes transfected by lentivirus plenti-CDNF was considerably higher than in astrocytes transfected by lentivirus plenti-his and the blank control (Fig. 1).

3.2. CDNF alleviates ER stress-induced astrocyte damage

The percentage of LDH released in the astrocyte + tunicamycin group was 11.79% ± 0.031, and in the plenti-his-transfected astrocyte + tunicamycin group was 13.84% ± 0.032; while the percentage of LDH released in the plenti-CDNF astrocyte + tunicamycin group was, significantly lower than the two former groups (7.52% ± 0.015) ($P < 0.01$). Meanwhile, the percentages of LDH released in the blank control (1.81% ± 0.014), in the plenti-his astrocyte (2.96% ± 0.015), and in the plenti-CDNF astrocyte (3.59% ± 0.011) groups were just as low as in the negative control groups (Fig. 2).

Table 1
Primers for real-time RT-PCR.

Genes	Sequences (5′-3′)	Length (bp)
IL-1ß	Sense: CATGGAATCCGTGTCTTCCT Antisense: GAGCTGTCTGCTCATTCACG	200
IL-6	Sense: ATCCAGTTGCCTTCTTGGGACTGA Antisense: TAAGCCTCCGACTTGTGAAGTGGT	133
TNF-α	Sense: CATCTTCTCAAAATTCGAGTGACAA Antisense: TGGGAGTAGACAAGGTACAACCC	175

3.3. CDNF suppresses secretion of inflammatory cytokines in astrocytes induced by ER stress

Five hours after the treatment of tunicamycin, inflammatory cytokine (IL-1ß, IL-6, and TNF-α) levels in an astrocyte culture medium were determined by ELISA. Tunicamycin significantly increased the release of IL-1ß in astrocytes (255.42 ± 37.36 pg/ml) and in plenti-his-transferred astrocytes (271.35 ± 40.22 pg/ml) after 5 h compared with astrocytes not treated with tunicamycin, and this was significantly attenuated in plenti-CDNF-overexpressed astrocytes (142.75 ± 27.55 pg/ml) ($P < 0.01$). The secretion of IL-6 was also stimulated by tunicamycin in astrocytes (414.06 ± 56.25 pg/ml) and in plenti-his-transferred astrocytes (391.55 ± 38.40 pg/ml), and this was significantly attenuated in plenti-CDNF astrocytes (312.74 ± 50.35 pg/ml) ($P < 0.05$). The secretion of TNF-α in the plenti-CDNF astrocyte + tunicamycin group (944.32 ± 156.96 pg/ml) was significantly lower than the astrocyte + tunicamycin (1573.51 ± 132.63 pg/ml) and the plenti-his-transferred astrocyte + tunicamycin (1634.85 ± 215.36 pg/ml) groups ($P < 0.01$) (Fig. 3).

3.4. CDNF alleviates the expression of pro-inflammatory cytokines in astrocytes induced by ER-stress

It is well-established that astrocytes participate in normal and abnormal processes of the CNS through the release of cytokines [3,4]. We therefore evaluated the expression of pro-inflammatory cytokines following ER-stress stimulation caused by tunicamycin (50 ng/ml) for 24 h. Real-time RT-PCR results showed that tunicamycin stimulation increased the mRNA expression levels of pro-inflammatory cytokines including IL-1ß, IL-6, and TNF-α, and plenti-CDNF transfection-attenuated tunicamycin-induced upregulation of mRNA expression of the three cytokines (Fig. 4).

4. Discussion

The purpose of the present study was to examine the effect CDNF overexpression has on ERS-induced cell damage and inflammatory cytokine release in astrocytes caused by tunicamycin. In ischemic reperfusion injury of the brain and spinal cord, astrocytes play an important role in the protection of neurons, neurogenesis and neuron repair [5,6]. Astrocytes can have an effect on the blood supply because their end feet are tightly attached to the blood vessels, which contribute to and maintain the functional integrity of the blood–brain barrier (BBB). Furthermore, they secrete an array of pro-inflammatory and anti-inflammatory cytokines to modify the microenvironment. Therefore, astrocyte [5,6] damage induced by ERS leads to the deterioration of neurons after ischemic reperfusion injury.

Previous evidence has also implicated the negative influence of neuroinflammation in a number of conditions of ischemic reperfusion injury. Inflammatory processes in the central nervous system (CNS) are mediated by the activation of glial cells capable of producing immunomodulatory molecules, phagocytosing cellular debris and recruiting immune cells from the periphery. Although activation of glia is essential for the maintenance of neuronal function following stress or insult, an uncontrolled response causes glial cell damage and is highly undesirable given the lack of regenerative capacity of the injured brain and spinal cord. Astrocytes, as the resident cells, play a crucial role in both physiological and pathological process in the CNS, by taking part in the maintenance of homeostasis, cell defense, and repair. Astrocytes provide structural, metabolic and trophic support for neurons, yet they are also immunocompetent cells capable of secreting inflammatory mediators [3]. The damage of astrocytes leads to excessive secretion of

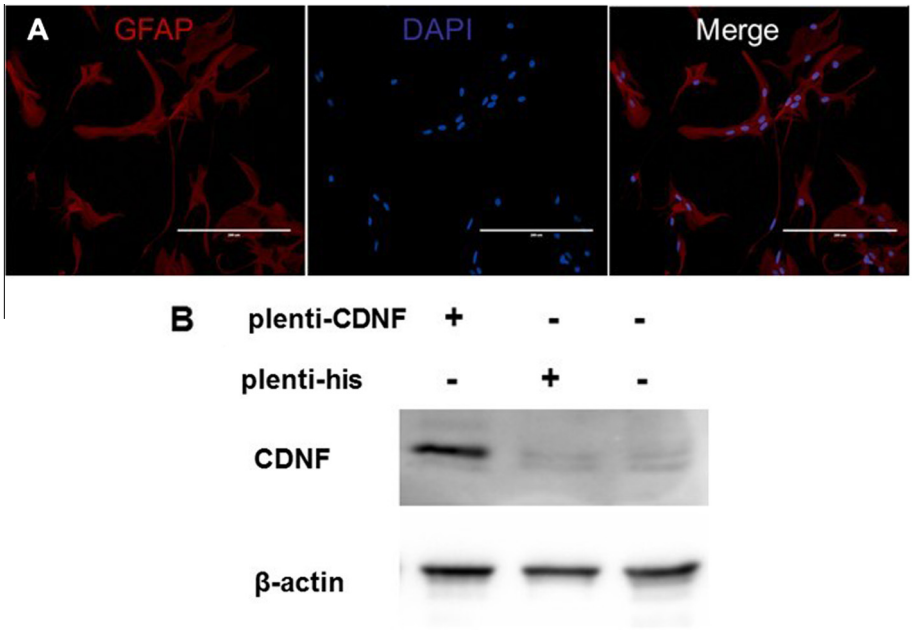

Fig. 1. Identified astrocytes and the expression of CDNF detected by Western blot. (A) More than 95% of the cells were GFAP positive, which means that they were identified as astrocytes. (B) The expression of CDNF protein in astrocytes transfected by lentivirus plenti-CDNF was considerably higher than astrocytes transfected by lentivirus plenti-his and the blank control.

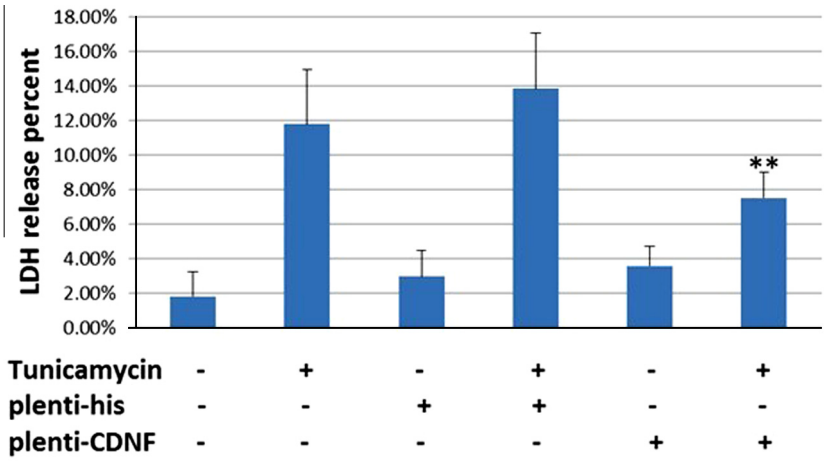

Fig. 2. LDH release percent measured by a LDH-Cytotoxicity Assay Kit. The percentages of LDH in the plenti-his and plenti-CDNF astrocyte were just as low as in the negative control group. The percentage of LDH released in the plenti-CDNF-transfected astrocyte + tunicamycin group was significantly lower than the astrocyte + tunicamycin and the plenti-his-transfected astrocyte + tunicamycin group ($^{**}P < 0.01$).

pro-inflammatory molecules such as IL-1β, IL-6 and TNF-α, which may have a deleterious impact on neuronal viability.

Prior studies have revealed that pathways activated by the ERS induce sterile inflammation. The ER is a multifunctional organelle involved in protein-folding and processing, intracellular Ca^{2+} homeostasis, and cell death signal-activation [4]. Disturbance of Ca^{2+} homeostasis and accumulation of unfolded proteins in the ER cause ER stress and activate the unfolded protein response (UPR) [17]. The UPR results in the phosphorylation and activation of PERK (PKR-like ER kinase), which triggers a cascade of events including phosphorylation of eIF2α, inhibition ofprotein synthesis, and activation of apoptotic signals [18–20]. There are three sensors of UPR: PERK, IRE1, and ATF6, all of which participate in upregulating inflammatory processes when pathways are activated by ERS [19]. According to recent research, ERS-induced UPR-signaling is associated with the production of many proinflammatory molecules [20]. The pathways of the ERS and the UPR have been reported in many recent reviews [21–23]. All three main branches of the UPR (i.e., those based on the activities of the proteins PERK, IRE1a, and ATF6) have been shown to mediate cell-autonomous proinflammatory transcriptional programs, which are mainly governed by transcription factors such as NF-κB [22,24]. NF-κB is one of the central mediators of proinflammatory pathways. Genes transcribed by NF-κB include those encoding crucial pro-inflammatory cytokines such as IL-1ß, IL-6, and TNF-α, and enzymes such as cyclooxygenase-2, involved in immunomodulation [10].

Furthermore, a highly complex inflammatory process called acute-phase response (APR) is also induced by the UPR in ERS. APR commences in the early phases of the innate immune response mainly due to the activity of proinflammatory factors such as IL-1ß, IL-6, and TNF-α [23]. The APR is not a single process but a group of organism-level physiological processes that are initiated soon after ERS. Some diagnostic signatures of the APR are activation of the production of various cytokines (e.g., IL-1ß and IL-6) and

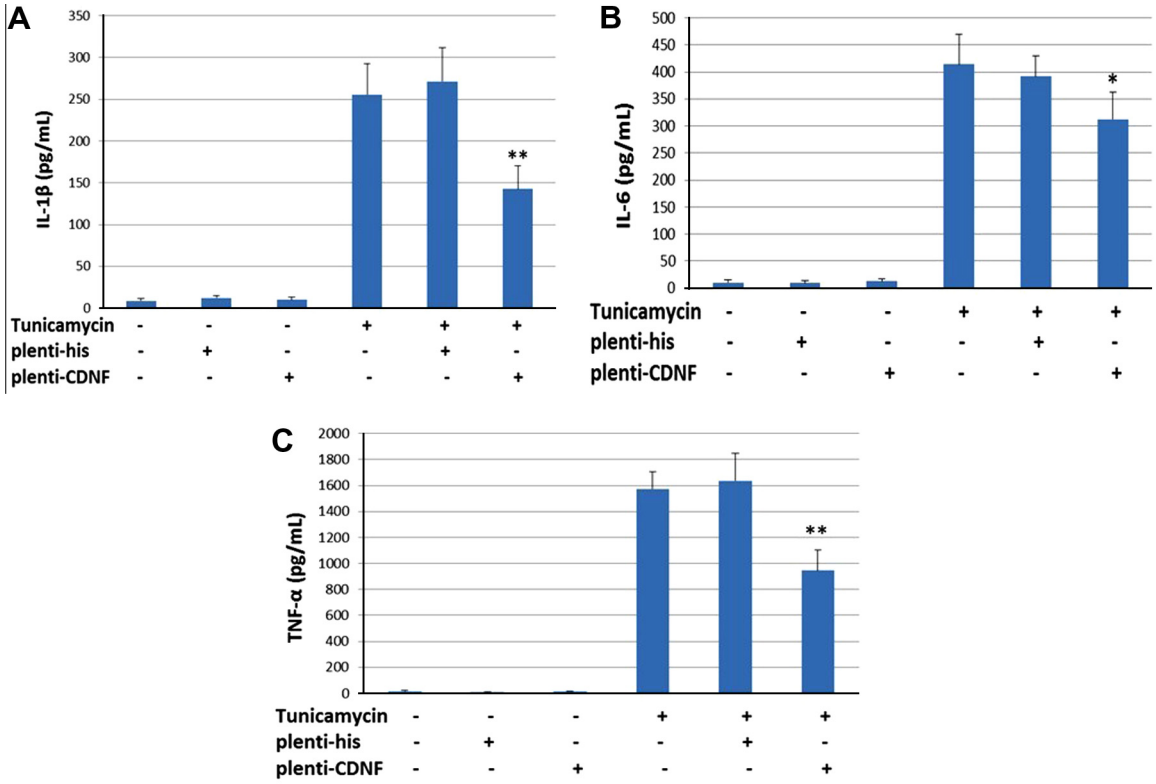

Fig. 3. Secretion of inflammatory cytokines in astrocytes detected by ELISA. (A) Tunicamycin significantly increased the release of IL-1ß in astrocytes and plenti-his-transferred astrocytes at 5 h compared with astrocytes without tunicamycin; this was significantly attenuated in plenti-CDNF-overexpressed astrocytes (**$P < 0.01$). (B) The secretion of IL-6 was stimulated by tunicamycin in the astrocytes and in the plenti-his-transfected astrocytes; this was significantly attenuated in plenti-CDNF astrocytes (*$P < 0.05$). (C) The secretion of TNF-α in the plenti-CDNF astrocyte + tunicamycin group was significantly lower than the astrocyte + tunicamycin and the plenti-his + tunicamycin group (**$P < 0.01$).

neutrophilia, which can aggravate tissue damage and cause more severe sterile immunopathological conditions [24].

In the CNS, astrocytes are crucial to providing protection and repair of the neurons insulted by ischemic reperfusion injury. Normal functioning of astrocytes requires the trafficking of large amounts of proteins through the ER, which makes them highly sensitive to perturbations in ER homeostasis. Any changes in this trafficking can lead to severe and chronic activation of the UPR, which in the long run might cause ERS-based inflammation and cell death. Tunicamycin, an UPR inducer, is used to induce the ERS, inflammation and cell death in astrocytes, imitating the process of astrocyte damage induced by the ERSR *in vitro*. In CNS ischemic reperfusion injury, astrocyte damage and inflammatory responses are induced by ERS caused by hypoxic-ischemia, glucose starvation, ATP depletion, oxidative stress and disturbance of Ca^{2+} homeostasis. Neurons insulted cannot be mediated by the protection from astrocytes, and CNS injury may be exacerbated due to the pro-inflammatory cytokines secreted by damaged astrocytes.

So far, there is no efficient method to prevent CNS ischemic reperfusion injury; however, conserved dopamine neurotrophic factor (CDNF) demonstrates a promising potential to resist ERS and protect astrocytes and neurons in the CNS. CDNF and MANF consist of two domains: an amino-terminal saposin-like domain that may interact with lipids or membranes, and a presumably unfolded carboxy-terminal domain that may protect cells against endoplasmic reticulum stress. Growing evidence suggests that MANF is an ER stress response protein and is able to protect cells against ER stress-induced cell death *in vitro*[13]. In line, the crystal structure suggests that MANF and CDNF may help protein folding in the ER. In the MANF C-terminal domain, the two cysteines in the ^{127}CKGC^{130} motif (^{132}CRAC^{135} in CDNF) form a C-terminal disulphide bridge that may facilitate the formation of cysteine bridges and protein folding in the ER, thus reducing the ER stress caused by unfolded or incorrectly folded proteins [12].

Therefore, in our study, we focused on the role of CDNF on ER stress-induced cell damage and tunicamycin-induced inflammation in astrocytes. We used a lentiviral-based system to overexpress CDNF in astrocytes. Our results show that the percentage of LDH released in the plenti-CDNF-transfected astrocyte + tunicamycin group was significantly lower than the control groups without CDNF overexpression, indicating that CDNF alleviates ER stress-induced astrocyte damage. Meanwhile, the secretion and mRNA expression levels of the pro-inflammatory cytokines IL-1ß IL-6, and TNF-α in astrocytes were increased by incubating the astrocytes in tunicamycin, and this upregulation was significantly suppressed by an overexpression of CDNF, demonstrating that CDNF plays an important role in astrocyte inflammation and functioning by resisting ER stress. Thus, CDNF overexpression may represent a promising strategy for neuroprotection in the CNS.

In summary, we have shown that effective expression of plenti-CDNF in primary astrocytes with the use of lentiviral vectors and the overexpression of CDNF in astrocytes give cells the potential to resist both ER stress and proinflammatory cytokine secretion. Whether CDNF provides the same protection from cell damage and an inflammatory response induced by ER stress to astrocytes *in vivo* remains to be seen. Future studies will explore CDNF gene delivery to astrocytes *in vivo* to advance research in animal models of neurologic disease.

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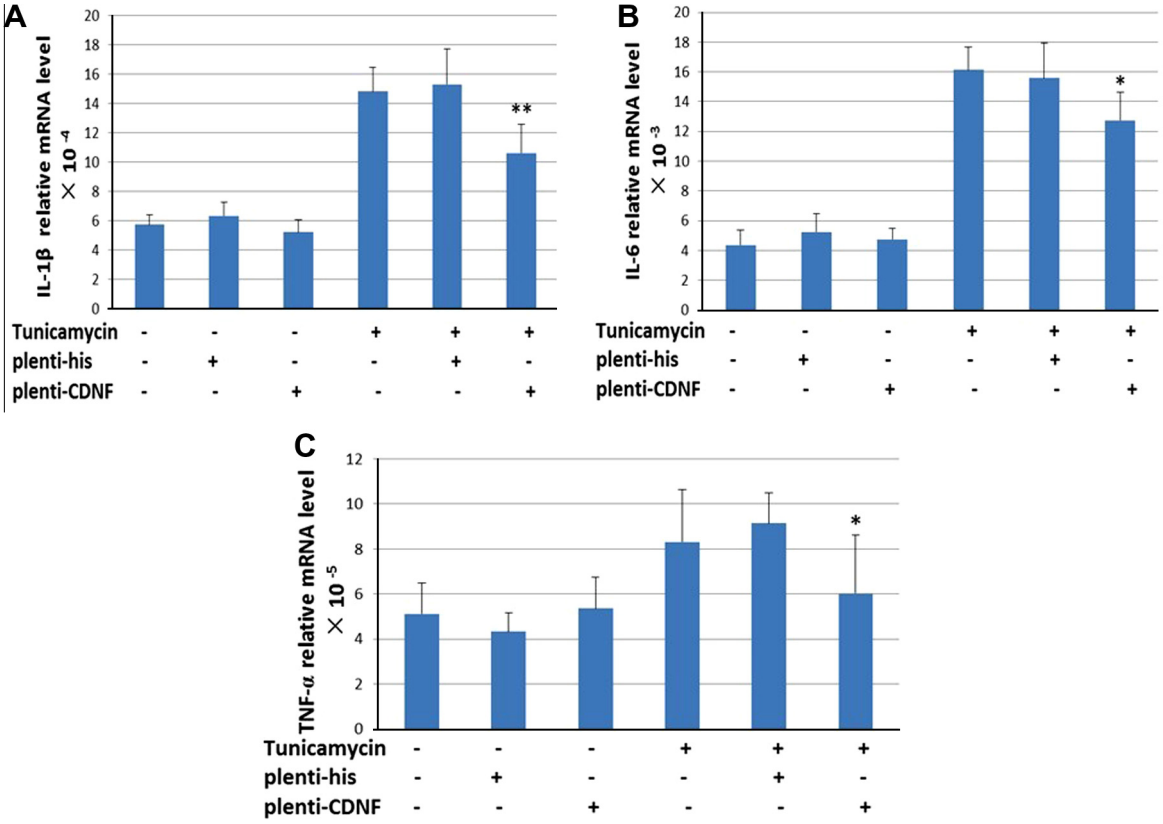

Fig. 4. Overexpression of plenti-CDNF attenuated tunicamycin-induced upregulation of mRNA expression of pro-inflammatory cytokines. (A) Tunicamycin significantly increased the relative IL-1ß mRNA levels in astrocytes and in plenti-his-transferred astrocytes at 24 h compared to astrocytes without tunicamycin, and this was significantly attenuated in the plenti-CDNF-overexpressed astrocytes (**$P < 0.01$). (B) The relative IL-6 mRNA levels were also stimulated by tunicamycin in astrocytes and in plenti-his astrocytes, and this was significantly attenuated in plenti-CDNF astrocytes (*$P < 0.05$). (C) The relative TNF-α mRNA levels in the plenti-CDNF astrocyte + tunicamycin group were significantly lower than the astrocyte + tunicamycin and plenti-his + tunicamycin groups (**$P < 0.01$).

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