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CART attenuates endoplasmic reticulum stress response induced by cerebral ischemia and reperfusion through upregulating BDNF synthesis and secretion

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ABSTRACT

Cocaine and amphetamine regulated transcript (CART), a neuropeptide, has shown strong neuroprotective effects against cerebral ischemia and reperfusion (I/R) injury *in vivo* and *in vitro*. Here, we report a new effect of CART on ER stress which is induced by cerebral I/R in a rat model of middle cerebral artery occlusion (MCAO) or by oxygen and glucose deprivation (OGD) in cultured cortical neurons, as well as a new functionality of BDNF in the neuroprotection by CART against the ER stress in cerebral I/R. The results showed that CART was effective in reducing the neuronal apoptosis and expression of ER stress markers (GRP78, CHOP and cleaved caspase12), and increasing the BDNF expression in I/R injury rat cortex both *in vivo* and *in vitro*. In addition, the effects of CART on ischemia-induced neuronal apoptosis and ER stress were suppressed by tyrosine receptor kinase B (TrkB) IgG, whereas the effects of CART on BDNF transcription, synthesis and secretion were abolished by CREB siRNA. This work suggests that CART is functional in inhibiting the cerebral I/R-induced ER stress and neuronal apoptosis by facilitating the transcription, synthesis and secretion of BDNF in a CREB-dependent way.

1. Introduction

Cerebral ischemia and reperfusion (I/R) injury is a common pathophysiological process in stroke [1]. Growing evidences demonstrate that endoplasmic reticulum (ER) stress markers, glucose-regulated protein 78 (GRP78), CATT/EBP homologous protein (CHOP), eukaryotic initiation factor 2α (eIF2α) and caspase12 are remarkably activated after cerebral I/R injury [2–6], and the inhibition of ER stress, either deletion of CHOP [7] or inhibition of eIF2α dephosphorylation [8], can attenuate the cerebral I/R injury.

A neuropeptide, cocaine and amphetamine regulated transcript (CART) is proven to show strong neuroprotective effects against the cerebral ischemic injury both *in vivo* and *in vitro* [9–12]. However, it has not been yet investigated whether CART can attenuate the ischemic neuronal injury by inhibiting the ER stress.

Our previous data have demonstrated that brain derived neurotrophic factor (BDNF) can mediate the functioning of CART on the survival of primary cultured hippocampal neurons [13], while it still remains unclarified whether BNDF can contribute to the neuroprotective effect of CART against ER stress response during cerebral I/R.

In this study, we investigated the effect of CART on the ER stress induced by cerebral I/R in rat or oxygen and glucose deprivation (OGD) in cultured cortical neurons, and explored the role of BDNF in neuroprotection of CART against the cerebral I/R-induced ER stress.

2. Materials and methods

2.1. Reagents

CART 55–102 was purchased from Phoenix Pharmaceuticals (Belmont, CA). Dulbecco's modified Eagle's medium (DMEM, high glucose), DMEM without glucose, neurobasal medium, OPTI-MEM I reduced serum medium, B27, L-glutamine, trizol, and cell apoptosis Kit for flow cytometry were purchased from Life Technologies (Gaithersburg, MD). Fetal bovine serum (FBS) was purchased from

Abbreviations: BDNF, brain derived neurotrophic factor; CART, cocaine and amphetamine regulated transcript; CHOP, CATT/EBP homologous protein; CREB, cAMP-response element binding protein; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; GRP78, glucose regulated protein 78; I/R, ischemia and reperfusion; MCAO, middle cerebral artery occlusion; OGD, oxygen and glucose deprivation; TrkB, tyrosine receptor kinase B.

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Hyclone (Logan, UT). Deoxyribonuclease I (DNase I), poly-L-lysine, and trypsin were purchased from Sigma–Aldrich (Saint Louis, MO). Turbofect siRNA transfection reagent, halt protease inhibitor cocktail, BCA kit, Revert Aid First-Strand cDNA Synthesis Kit, and Maxima SYBR Green qPCR Master Mix were purchased from Thermo Fisher Scientific (Rockford, IL). Rat anti-GRP78, rabbit anti-CHOP, and rat anti-caspase12 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-CREB, and rabbit anti-α-tubulin were purchased from Cell Signalling Technology (Beverly, MA). Rabbit anti-BDNF, and rabbit anti-TrkB IgG were purchased from Abcam (Cambridge, UK). Horseradish peroxidase-conjugated secondary antibodies were purchased from Zhongshan Goldenbridge Biotechnology Co. (Beijing, China). Non-fat milk (NFM) was purchased from BD (Franklin Lakes, NJ). ECL western blotting reagent kits, and BDNF sandwich ELISA Kit were purchased from Millipore (Billerica, MA). Lysis buffer, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Beyotime (Jiangsu, China).

2.2. Animals

Pregnant Sprague–Dawley (SD) rats and SD male rats (280–300 g) were obtained from the Department of Laboratory Animal Science of Peking University Health Science Center (Beijing, China). All animal care and use protocols as set out in the guidelines of the Animal Care and Use Committee of Peking University were followed.

2.3. Transient MCAO model and measurement of infarct volume

The transient middle cerebral artery occlusion (MCAO) rat model was performed using the intraluminal suture technique as previously described [18]. The CART, dissolved in 0.5 ml normal saline and dosed by 1 nM/kg, was administered through vena caudalis after onset of ischemia. Normal saline was given to sham and MCAO rats. The infarct volume is expressed as the percentage of (volume of unlesion hemisphere – uninfarct volume of lesion hemisphere) per volume of unlesion hemisphere.

2.4. Primary cortical neuron cultures

Primary cultures of cortical neurons were prepared from the cortex of E18 rat embryos following previously described with minor modification [13]. The cells were plated on the poly-L-lysine coated dishes at the density of 70,000 cells/cm^2 and cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. After 4 h of attachment, the medium was totally changed to neurobasal medium supplemented with 2% B27 and 1% L-glutamine. Afterwards, the medium was half replaced by neurobasal medium supplemented with B27 and L-glutamine every 3 days. Cultures were used at Day 7 *in vitro*.

2.5. Oxygen and glucose deprivation

Cultured rat cortical neurons were incubated with DMEM without glucose at 37 °C for 4 h in an anaerobic jar (UniTech Bioscience, Guangzhou) that is supplemented with a humidified gas mixture containing 95% N_2 and 5% CO_2. After 4 h of OGD, the medium was replaced by neurobasal medium and cells were further incubated at 37 °C in a humidified 95% air and 5% CO_2 with different times of reoxygenation for individual experiments. For control experiments, cells were cultured in DMEM with 10% FBS in a normoxic (21% O_2) environment at 37 °C and replaced by neurobasal medium after 4 h. The CART and the antagonists were maintained before and during the OGD injury, depending on each experiment.

2.6. siRNA transfection

Seven days after plating, the cell culture medium was replaced by the OPTI-MEM I medium for 12 h and the neurons were transfected with siRNA in a final concentration of 50 nM using Turbofect siRNA transfection reagent according to the manufacturer's protocol. The rat CREB siRNA was purchased from GenePharma (Shanghai), with the oligonucleotide sequences as follows: CREB siRNA, sense 5′-GCCAAGGAUUGAAGAAGAATT-3′, antisense 5′-UUCUUCUUCAAUCCUUGGCTT-3′; scrambled siRNA, sense 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense 5′-ACGUGACACGUUCGGAGAATT-3′.

2.7. Measurement of cell apoptosis by flow cytometry

Cell apoptosis was assessed by flow cytometry using dead cell apoptosis Kit according to the manufacturer's protocol. After the treatment as indicated in each experiment, which was followed by OGD for 4 h and reoxygen for 24 h, the neurons were harvested and incubated with annexin V and PI. Then stained cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences).

2.8. Western blotting analysis

The cortex tissue in peri-infarct (penumbra) or cortical neurons were harvested on ice in a lysis buffer plus 1 mM PMSF and 1:100 volume of halt protease inhibitor cocktail. The protein (25 μg) was separated by SDS–PAGE, and transferred to a PVDF membrane. The membrane was blocked with 5% skim milk in Tris buffer saline (TBS) Tween-20 for 1 h and then incubated overnight at 4 °C with the primary antibody. The blots, after being washed with TBST, were incubated with horseradish peroxidase-conjugated secondary antibodies (1:6000) for 1 h at room temperature, whereas ECL reagent kits were used for protein detection. The signals were quantified using quantity one (Bio-Rad), and the values were normalized to α-tubulin signal and presented as the mean ± SEM.

2.9. Quantity real-time PCR

After the treatment as indicated in each experiment, which was followed by OGD for 4 h and reoxygen for 4 h, the mRNA was isolated with the trizol reagent. Real-time PCR was conducted in a 25 μl volume with real-time PCR kits in Mx3000 multiplex quantitative PCR system (Stratagene, La Jolla, CA, USA). Amplification was performed using an initial 10 min step at 95 °C, followed by 35 cycles of 94 °C for 30 s, annealing at 60 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min for elongation. β-Actin served as an internal control. The primers used in this study were sequenced as follows: rat BDNF, sense 5′-CGACGTCCCTGGCTGACACTTTT-3′, antisense 5′- AGTAAGGGCCCGAACATACGATTGG-3′; rat β-actin, sense, 5′-TCACCAACTGGGACGATA-3′, antisense, 5′-AGGCATACAGGGACAACA-3′.

2.10. Enzyme-linked immunosorbent assay (ELISA)

BDNF concentration in the culture medium was determined by a BDNF sandwich ELISA Kit according to the manufacturer's instructions. Samples were measured by the absorbance at 450 nm with a microplate reader. The range of detection was 7.8–500 pg/ml.

2.11. Statistical analysis

All values are presented as mean ± standard error of the mean (SEM). Comparisons among multiple groups involve one-way analysis of variance (ANOVA) by Newman–Student–Keuls test.

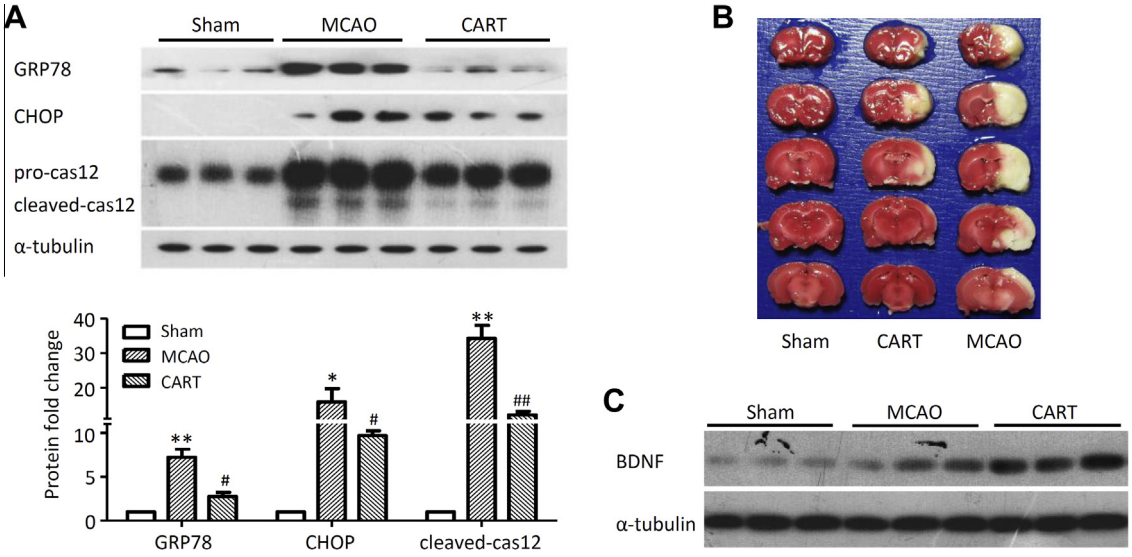

Fig. 1. The effects of CART on ER stress molecules, infarct volume and BDNF expression in rat brain. (A) The expression of GRP78, CHOP and caspase12 was analyzed by Western blotting. $n = 3$. (B) TTC staining of rat brain slice. White is infarct area and red is normal area. $n = 10$. (C) BDNF expression in cortical tissues was determined by Western blotting. $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Sham; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. MCAO. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

GraphPad Prism (GraphPad Software Inc., San Diego, CA) was used in all analyses. P values less than 0.05 were considered to be significant.

3. Results

3.1. CART attenuates ER stress response, reduces infarct volume and promotes BDNF expression in rat brain of MCAO

The ER stress response, BDNF expression and neuroprotective effect of CART were observed in rat brain during I/R injury. Western blotting results indicated that MCAO significantly increased the expression of GRP78, CHOP and cleaved-caspase12 compared with Sham group. However, treatment with CART inhibited the high expression of these three ER molecules (Fig. 1A). With regards to the cerebral infarct volume, it was induced in MCAO group (63.29% ± 3.03%, $P < 0.001$ vs. Sham). CART treatment dramatically decreased the volume induced by MCAO (29.24% ± 3.49%, $P < 0.001$ vs. MCAO) (Fig. 1B). Moreover, an increase in BDNF expression was observed in cortical tissues in both of the MCAO (2.15 ± 0.75-fold, $P < 0.05$) and the CART group (3.44 ± 0.69-fold, $P < 0.01$) compared with Sham group, but the expression between the CART and the MCAO group reached significantly difference ($P < 0.05$, Fig. 1C).

3.2. CART attenuates ER stress response, inhibits apoptosis and promotes BDNF expression in OGD treated rat cortical neurons

To investigate the neuroprotective effect of CART on OGD induced injury, cortical neurons were treated with different doses

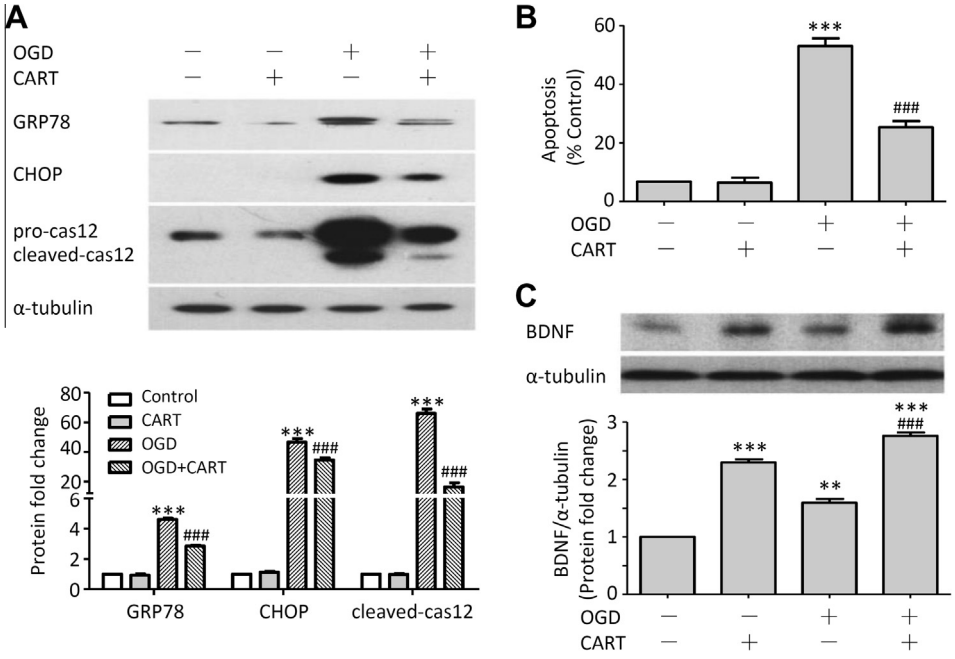

Fig. 2. The influence of CART on ER stress molecules, apoptosis and BDNF expression in cultured cortical neurons. (A) Cortical neurons were treated with CART for 30 min, followed by OGD for 4 h and reoxygen for 24 h, and then the proteins were harvested. The expression of GRP78, CHOP and caspase12 was analyzed by Western blotting. (B) Cell apoptosis was detected by flow cytometry. (C) Cortical neurons were treated with CART for 30 min, followed by OGD for 4 h and reoxygen for 8 h. BDNF protein was determined by Western blotting. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Control; $^{\#\#\#}P < 0.001$ vs. OGD. $n = 3$.

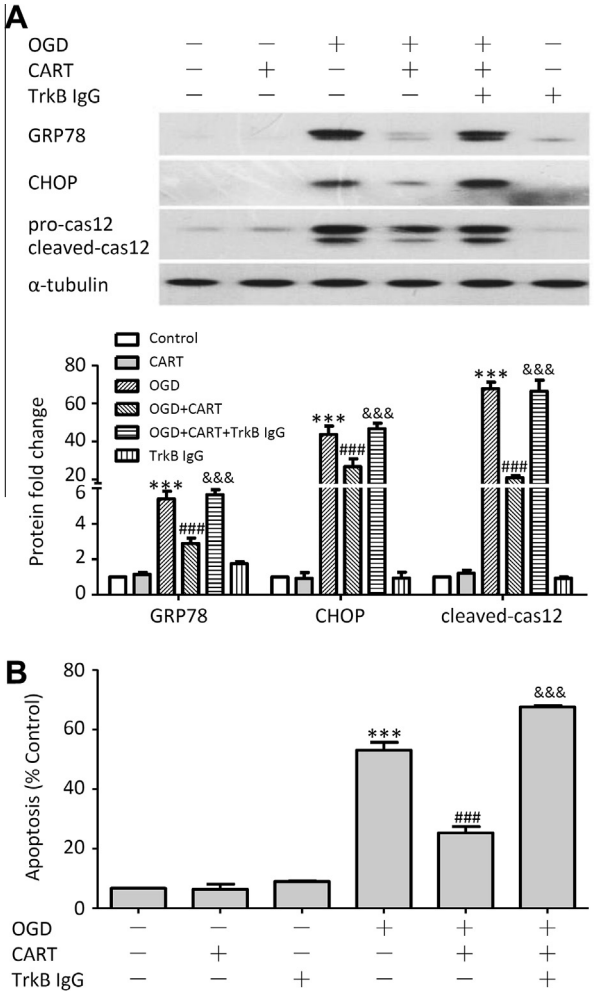

Fig. 3. BDNF mediates the role of CART on ER stress and apoptosis induced by OGD. Cortical neurons were pretreated with 5 μg/ml TrkB IgG for 30 min and then treated with 10 nM CART for 30 min followed by OGD for 4 h and reoxygen for 24 h. (A) The expression of GRP78, CHOP and caspase12 was analyzed by Western blotting. (B) Neuronal apoptosis was determined by flow cytometry. $^{***}P < 0.001$ vs. Control; $^{\#\#\#}P < 0.001$ vs. OGD; $^{\&\&\&}P < 0.001$ vs. OGD + CART. $n = 3$.

of CART for 30 min, followed by exposure to OGD for 4 h and reoxygen for 24 h. The MTT results showed that cell viability was significantly reduced by OGD, which was inhibited by both 1 and 10 nM CART (data not shown). Therefore, the 10 nM of CART was used in the subsequent experiments. Western blotting results showed that OGD induced a higher expression of GRP78, CHOP and cleaved-caspase12, while the CART pretreatment attenuated the expression of three ER stress markers (Fig. 2A). Moreover, the neuronal apoptosis was significantly induced by OGD, but inhibited by CART pretreatment (Fig. 2B). In addition, the BDNF expression was much higher in CART pretreated group than in OGD group (Fig. 2C).

3.3. BDNF mediates the inhibitory effects of CART on OGD-induced ER stress and apoptosis

To verify the role of BDNF on the ER stress inhibition by CART, the BDNF receptor (tyrosine receptor kinase B) antibody (TrkB IgG, 5 μg/ml) was used to block the function of BDNF receptor. The results showed that CART treatment inhibited the expression of ER stress markers, which was blocked by TrkB IgG (Fig. 3A), and the anti-apoptosis effect of CART was also abolished by TrkB IgG (Fig. 3B).

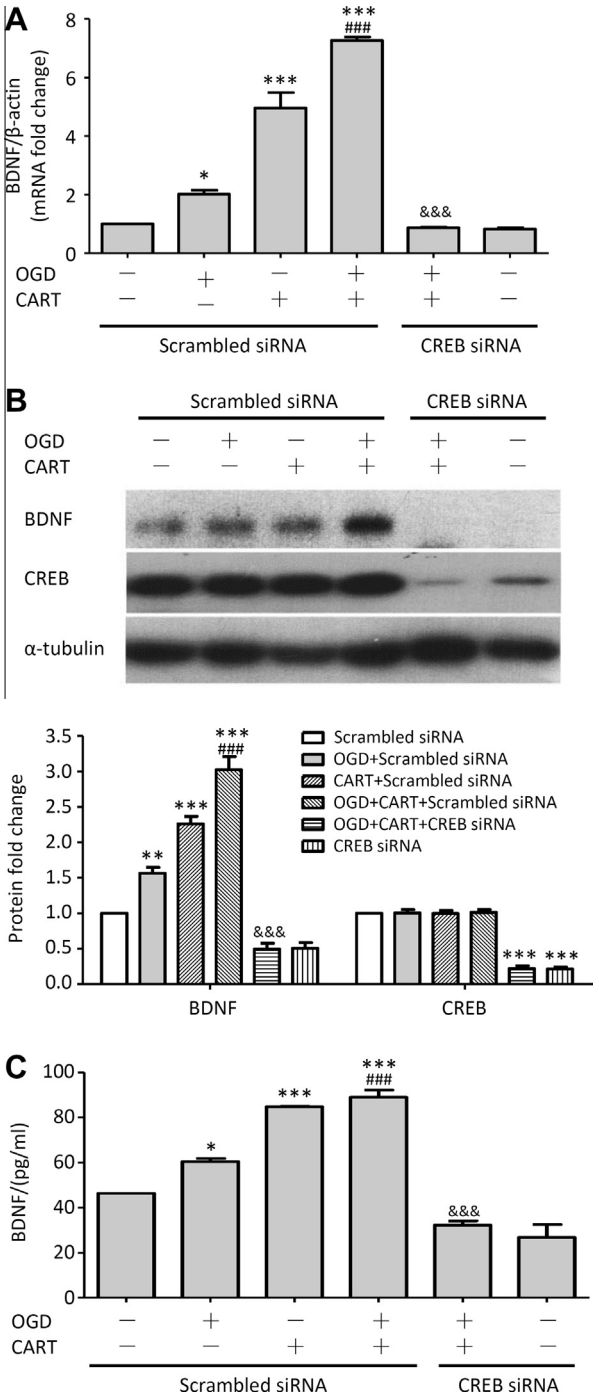

Fig. 4. CART promotes BDNF transcription, synthesis and secretion through CREB. (A) BDNF mRNA was quantified by real-time PCR. (B) BDNF and CREB protein levels were determined by Western blotting. (C) The content of BDNF in medium was analyzed by ELISA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. scrambled siRNA; $^{\#\#\#}P < 0.001$ vs. OGD + scrambled siRNA; $^{\&\&\&}P < 0.001$ vs. OGD + CART + scrambled siRNA. $n = 3$.

3.4. CREB mediates the effect of CART on BDNF upregulation

CREB siRNA or scrambled siRNA were transfected into neurons, aiming to verify whether cAMP-response element binding protein (CREB) mediated the BDNF upregulation induced by CART or not. In our previous work, BDNF mRNA reached the peak at 8 h and BNDF protein at 12 h after treatment of CART. Therefore, the mRNA was collected after treatment of OGD for 4 h and reoxygen for 4 h, and the protein and the culture medium were harvested after

treatment of OGD for 4 h and reoxygen for 8 h, the same as previous work. The data revealed that the protein level of CREB was inhibited by CREB siRNA (Fig. 4B). Besides, the BDNF mRNA level (Fig. 4A) and protein level both in cells (Fig. 4B) and medium (Fig. 4C) were decreased in CREB siRNA transfected neurons with or without CART treatment, while scrambled siRNA had no effect.

4. Discussion

The ER stress is a pathological process of imbalance in ER homeostasis and physical function disorder [14]. The three ER stress markers, GRP78, CHOP, and caspase12, are activated during ER stress [15]. A growing body of evidence suggested that the ER stress has been implicated in the pathogenesis of cerebral I/R injury [2,6,8,16]. In present study, we found that CART significantly downregulated the expression of GRP78 and CHOP, and inhibited the cleavage of caspase12 both *in vivo* and *in vitro*. Our results strongly support the finding that CART inhibited ER stress response and reduced neuronal apoptosis during cerebral I/R. It has been known that BDNF, a member of neurotrophin family, plays a beneficial role in stroke [17–19]. Recent studies indicate that CART promotes the survival, proliferation, migration and differentiation through BDNF upregulation [13,20–22]. However, it has not been investigated as to whether the BNDF mediated the protective effect of CART against ER stress during cerebral I/R. Our results showed that CART significantly promoted the expression of BDNF *in vivo* and *in vitro*. Although the expression of BDNF was lightly increased in rat brain with MCAO and OGD-treated neurons, it was not enough to provide neuroprotection against neuronal injury. The present results also indicated that the inhibitory effects of CART on OGD-induced ER stress and apoptosis in cortical neurons were blocked by antibody to the BDNF receptor (TrkB). These data suggest that BDNF mediated the neuroprotective effect of CART. Since the transcription factor CREB is involved in BDNF production in neurons [23,24], it is possible that the BDNF upregulation by CART is CREB-dependent. Our results indicate that pre-treatment with CREB siRNA significantly suppressed CART-induced BDNF in cultured cortical neurons.

Taken together, the present data suggest that CART inhibited cerebral I/R-induced ER stress and neuronal apoptosis by upregulating BDNF in a CREB-dependent way. These findings have provided a new sight in molecular mechanism on the neuroprotective effect of CART on brain ischemia.

Acknowledgments

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