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Cold-inducible RNA-binding protein mediates neuroinflammation in cerebral ischemia

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ABSTRACT

Background: Neuroinflammation is a key cascade after cerebral ischemia. Excessive production of proinflammatory mediators in ischemia exacerbates brain injury. Cold-inducible RNA-binding protein (CIRP) is a newly discovered proinflammatory mediator that can be released into the circulation during hemorrhage or septic shock. Here, we examine the involvement of CIRP in brain injury during ischemic stroke.
Methods: Stroke was induced by middle cerebral artery occlusion (MCAO). *In vitro* hypoxia was conducted in a hypoxia chamber containing 1% oxygen. CIRP and tumor necrosis factor-α (TNF-α) levels were assessed by RT-PCR and Western blot analysis.
Results: CIRP is elevated along with an upregulation of TNF-α expression in mouse brain after MCAO. In CIRP-deficient mice, the brain infarct volume, induction of TNF-α, and activation of microglia are markedly reduced after MCAO. Using microglial BV2 cells, we demonstrate that hypoxia induces the expression, translocation, and release of CIRP, which is associated with an increase of TNF-α levels. Addition of recombinant murine (rm) CIRP directly induces TNF-α release from BV2 cells and such induction is inhibited by neutralizing antisera to CIRP. Moreover, rmCIRP activates the NF-κB signaling pathway in BV2 cells. The conditioned medium from BV2 cells exposed to hypoxia triggers the apoptotic cascade by increasing caspase activity and decreasing Bcl-2 expression in neural SH-SY5Y cells, which is inhibited by antisera to CIRP.
Conclusion: Extracellular CIRP is a detrimental factor in stimulating inflammation to cause neuronal damage in cerebral ischemia.
General significance: Development of an anti-CIRP therapy may benefit patients with brain ischemia.

1. Introduction

Stroke is the third leading cause of death in the Western world and the most frequent cause of permanent disability in adults worldwide [1]. Currently, therapeutic options for treating patients with ischemic stroke are extremely limited. Recombinant tissue-plasminogen activator for thrombolysis is the only approved specific treatment for this devastating illness. It is limited however, by its short therapeutic window (within 3 h) and side effects [1]. In order to develop new neuroprotectant therapies, understanding the complex pathogenesis of brain ischemia and finding new drug targets are imperative.

The mammalian brain is vulnerable to various environmental and pathophysiological insults. When a stroke occurs, the brain is susceptible to hypoxic or ischemic insults. Many studies have shown that inflammation contributes to the pathogenesis of brain injury in stroke by triggering numerous cellular and molecular events that cause neuronal damage [1,2]. In the periphery, immune cells such as leucocytes (neutrophils, monocytes, and macrophages), B cells, and T cells can access most organs in response to inflammation [3,4]. However, due to the blood–brain barrier, most systemic immune cells cannot reach the brain. Microglia are resident macrophages within the central nervous system and are responsible for the majority of inflammatory activity in the brain [4]. When microglia detect invading pathogens or tissue injury, they become 'activated', and start proliferating, migrating, phagocytizing, and producing proinflammatory cytokines and oxidants, leading to neuronal damage [2,3,5].

Cold-inducible RNA-binding protein (CIRP) is the first identified cold shock protein in mammalian cells. The protein sequences of CIRP in mouse and rat are identical, while human CIRP has 95% homology to them [6]. CIRP in both murine and human is a 172-amino-acid nuclear protein consisting of one amino-terminal consensus sequence RNA-binding domain and one carboxyl-terminal glycine-rich domain [6,7]. CIRP is thought to modulate gene expression during mild hypothermia by functioning as an RNA chaperone to facilitate translation [8]. CIRP is constitutively but weakly expressed in various tissues [9,10]. In addition to cold stress, CIRP expression is increased in response to UV irradiation

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[11] and hypoxia [12]. It has been reported that CIRP can be detected in the brain and its expression increases under ischemic conditions [13]. Our recent study also shows that CIRP increases significantly after hemorrhagic and septic shock in animals, and administration of recombinant murine CIRP (rmCIRP) in healthy animals causes organ injury [14].

Based on the information above, we hypothesized that CIRP would contribute to brain inflammation and lead to neuronal damage in cerebral ischemia. In this study, we first determined the expression of CIRP and proinflammatory cytokine tumor necrosis factor-α (TNF-α) in mice after cerebral ischemia induced by middle cerebral artery occlusion (MCAO). Then, we compared brain infarct size and activation of microglia between wild type and CIRP-deficient mice after MCAO. We further applied an *in vitro* cell culture system to elucidate the role of CIRP in regulating inflammation and the signaling pathway in mediating CIRP activity in microglia after exposure to hypoxia. Finally, we assessed CIRP's effects on neural cell death.

2. Materials and methods

2.1. Experimental animals and model of cerebral ischemia

CIRP-deficient (*Cirbp*$^{-/-}$) mice with C57BL/6 background were provided by Kumamoto University in Japan. Male age matched C57BL/6 mice (20–25 g) were purchased from Taconic (Albany, NY). All mice were housed in a temperature controlled room on a 12 h light-to-dark cycle and fed a standard laboratory diet. The animals were allowed to acclimate for at least 5 days under these conditions before being used for experiments. All animal experiments were carried out in accordance with the National Institutes of Health guidelines for the use of experimental animals. The project was approved by the Institutional Animal Care and Use Committee (IACUC) of the Feinstein Institute for Medical Research.

Mice were fasted overnight but had access to water *ad libitum* before induction of cerebral ischemia. Permanent focal cerebral ischemia was induced by MCAO as previously described by Belayev et al. [15], with some modifications. Briefly, anesthesia was induced by isoflurane inhalation and subsequently maintained by intravenous boluses of pentobarbital (15 mg/kg BW). Body temperature was maintained at 37 °C using a heating pad with a temperature monitor (Harvard Apparatus, Holliston, MA). The right common carotid artery (CCA) was exposed through a ventral midline neck incision and was carefully dissected free from the vagus nerve and fascia, from its bifurcation to the base of the skull. The distal branches of the external carotid artery (ECA) were then dissected, ligated and divided to create an ECA stump. The internal carotid artery (ICA) was isolated and separated from the adjacent vagus nerve and the pterygopalatine artery was dissected and ligated close to its origin. Then, a 1-cm length of 7-0 poly-L-lysine coated monofilament nylon suture was inserted through the proximal ECA into the ICA and advanced to the middle cerebral artery (MCA) origin to occlude it. The silk suture around the ECA stump was tightened around the intraluminal nylon suture to prevent bleeding. Occlusion of the MCA was ascertained by inserting the suture to a pre-determined length of 8–10 mm from the carotid bifurcation and feeling for resistance as the suture tip approached the proximal anterior cerebral artery. The cervical wound was then closed in layers and mice were allowed to recover from anesthesia. The intraluminal suture was left *in-situ* and mice were allowed unrestricted access to food and water. The sham-operated animals had the same procedures except for the MCA occlusion. The 48 h post sham-operation group was used as a sham control. At 30 and 48 h post-operation, the animals were sacrificed and brain tissue from the infarcted hemisphere was collected for various analyses. The induction of stroke by the MCAO procedure was confirmed by staining the brain with 1.5% triphenyl tetrazolium chloride (TTC) at 37 °C for 30 min and then immersing it in 10% formalin overnight.

2.2. Immunohistochemistry analysis of the brain sections

Paraffin sections of brain tissue were de-waxed and rehydrated. Slides were soaked in 20% citric acid pH 6.0 buffer (Vector Labs, Burlingame, CA) and heated in the microwave oven and maintained at 95 °C for 15 min for antigen retrieval. Endogenous peroxidase was blocked by 2% H_2O_2 in 60% methanol for 20 min. Normal goat serum (3%) was used to block the nonspecific binding sites. The sections were then incubated with rabbit anti-allograft inflammatory factor 1 (AIF1) primary antibodies (1:100, Proteintech Group, Chicago, IL) overnight at 4 °C, followed by biotinylated anti-rabbit IgG (1:200, Vector Labs) for 1 h. Vectastain ABC reagent and DAB kit (Vector Labs) were used to reveal the immunohistochemical reaction and counterstained with hematoxylin. The primary antibody was substituted with normal rabbit IgG as the negative control. The immunostaining was examined under a Nikon Eclipse E600 microscope.

2.3. BV2 cell culture and exposure to hypoxia

Murine microglial BV2 cells were obtained from Dr. Philippe Marambaud at The Feinstein Institute for Medical Research and are a reliable model for studying the biology of primary microglia [16,17]. BV2 cells have been used to study the cellular responses of microglia to hypoxic stress [18,19]. BV2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 37 °C incubator with 5% CO_2. To simulate the *in vivo* hypoxic condition, BV2 cells were cultured in a sealed chamber containing 1% O_2, 5% CO_2 and 94% N_2 for 20 h or 30 h.

2.4. Determination of gene expression by real-time polymerase chain reaction (PCR)

Total RNA was extracted from brain tissues and BV2 cells using the TRLzol reagent (Invitrogen). Real-time PCR was carried out on cDNA samples which were reversely transcribed from 2 μg RNA by using murine leukemia virus reverse-transcriptase (Applied Biosystems, Foster City, CA). A PCR reaction was carried out in 24 μl of the final volume containing 0.08 μmol of each forward and reverse primer, 2 μl cDNA, 9.2 μl H_2O and 12 μl Power SYBR Green PCR Master Mix (Applied Biosystems). Amplification was conducted in an Applied Biosystems 7300 real-time PCR machine under the thermal profile of 50 °C for 2 min, 95 °C for 10 min and followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The level of mouse β-actin mRNA was used for normalization and each specific mRNA was conducted in duplicate. Relative expression of mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method and results were expressed as fold change in comparison with the control group. The sequences of primers used in PCR are the following: CIRP (NM_007705) forward: 5′-AGC TCG GGA GGG TCC TAC AG-3′ and reverse: 5′-GAG GGC TTT TAC TCG TTG TGT GT-3′; TNF-α (X02611) forward: 5′-AGA CCC TCA CAC TCA GAT CAT CTT C-3′ and reverse: 5′-TTG CTA CGA CGT GGG CTA CA-3′; β-actin (NM_007393) forward: 5′-CGT GAA AAG ATG ACC CAG ATC A-3′ and reverse: 5′-TGG TAC GAC CAG AGG CAT ACA G-3′. A dissociation curve was performed for each analysis to confirm the specificity of the PCR product.

2.5. Western blot analysis of protein expression

Brain tissues and BV2 cells were lysed with RIPA buffer containing a protease inhibitor cocktail (Roche Applied Science; Indianapolis, IN). The protein concentration of the lysate was determined by the DC protein assay kit (Bio-Rad, Hercules, CA). 30 μg of protein was subjected to a 4–12% Bis–Tris gel electrophoresis in MES-SDS running buffer (Invitrogen). After electrophoresis, the gel was transferred onto a 0.2-μm nitrocellulose membrane and then blocked with 0.1% casein in 10 mM phosphate buffer, pH 7.5. Then, the membrane was incubated

with anti-CIRP rabbit polyclonal antibody (1:200; ProteinTech Group), anti-TNF-α mouse monoclonal antibody (1:250; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Bcl-2-polyclonal rabbit antibody (1:500; Santa Cruz Biotechnology) overnight at 4 °C, followed by incubation with infrared dye-linked anti-rabbit or anti-mouse IgG (1:15,000; LI-COR Biosciences, Lincoln, NE) for 1 h. The membranes were subjected to the Odyssey infrared image system (LI-COR Biosciences) and densities were analyzed and calculated. To verify the equal protein loading, the same membrane was stripped with 0.1 M HCl and then re-blotted with β-actin antibody (1:10,000; Sigma-Aldrich, St. Louis, MO).

2.6. Identification of CIRP translocation in BV2 cells

A plasmid containing murine CIRP coding region fused with green fluorescence protein (GFP) was obtained from Dr. Jun Fujita (Kyoto University, Japan). The plasmid was transfected to BV2 cells by lipofectamine (Invitrogen), according to the manufacturer's instruction. At 24 h after transfection, cells were exposed to hypoxia for another 24 h. Then, the cellular location of GFP-CIRP expression in BV2 cells was detected by an inverted fluorescence microscope.

2.7. Measurement of extracellular CIRP and TNF-α protein

The conditioned medium from BV2 cells was incubated with 28.6% trichloroacetic acid (Sigma-Aldrich) at 4 °C overnight, then centrifuged at 14,000 *g* for 10 min to precipitate the protein. The precipitated protein from 1 ml conditioned medium was washed with fresh cold acetone and subjected to Western blotting for the determination of CIRP levels. Levels of TNF-α in the cultured medium were measured by using an enzyme-linked immunosorbent assay (ELISA) kit specific for mouse TNF-α (BD Biosciences, San Jose, CA), according to the manufacturer's instruction.

2.8. Measurement of NF-κB activation

A plasmid containing NF-κB binding elements in the promoter region and a luciferase as reporter gene obtained from Promega (Madison, WI) were transfected to BV2 cells. At 24 h after transfection, rmCIRP or LPS was added to the cells for another 24 h. Cells were lysed and luciferase activity was measured using a luciferase activity kit (Promega). The luciferase activity was expressed as relative light unit (RLU) per mg protein of total cell lysate.

2.9. Culture and differentiation of SH-SY5Y

Human neuroblastoma SH-SY5Y cells (ATCC, Manassas, VA) were cultured in RPMI-1640 medium (Invitrogen) containing 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. SH-SY5Y cells were treated with retinoic acid (10 μM, Sigma-Aldrich) for 7 days to be differentiated into neurons [20]. The rmCIRP and antisera to CIRP were prepared as described previously [14]. Conditioned medium from BV2 cells with or without exposure of hypoxia and with or without co-administration of antisera to CIRP was collected and filtered through a 0.22 μm filter. Then, the conditioned medium was added to SH-SY5Y cells.

2.10. Determination of activation of the apoptotic cascade

Caspase activity in differentiated SH-SY5Y cells was determined by using a fluorimetric assay kit (Roche), according to the manufacturer's instruction. This assay kit can detect various caspase activities, including caspase-3, -6, -7, and -8. The levels of anti-apoptotic protein Bcl-2 in differentiated SH-SY5Y cells were determined by Western blotting.

2.11. Statistical analysis

All data are expressed as means ± standard errors (SE) and compared by one-way analysis of variance (ANOVA) and Student–Newman–Keuls (SNK) test or Student's *t*-test by comparing two groups. Differences in values were considered significant if $P < 0.05$.

3. Results

3.1. Induction of CIRP and TNF-α in mouse brain after MCAO

To examine whether CIRP was regulated under ischemic stroke, we measured the expression levels of CIRP after MCAO. CIRP gene expression increased by 2.7- and 2.6-fold at 30 h and 48 h after MCAO respectively, compared to sham animals ($P < 0.05$; Fig. 1A). Cellular protein levels of CIRP also increased by 1.6- and 2.1-fold at 30 h and 48 h after MCAO, respectively ($P < 0.05$; Fig. 1B). The levels of TNF-α gene expression were elevated 5.3- and 27.3-fold at 30 h and 48 h after MCAO respectively, compared to sham animals ($P < 0.05$; Fig. 1C). TNF-α protein expression increased 2.5- and 2.0-fold after 30 and 48 h after MCAO, respectively ($P < 0.05$; Fig. 1D). We observed a discrepancy in the fold-increase between TNF-α mRNA and protein levels in the brain tissues, which could be due to its release to extracellular space and was not detected well by Western blotting after sample preparation. Nevertheless, these results indicated that CIRP expression was elevated and accompanied with the increase of TNF-α expression during stroke.

3.2. Effect of CIRP deficiency on infarct volume and activation of microglia after MCAO

To identify the role of CIRP in stroke, wild type and *Cirbp*$^{-/-}$ mice were subjected to MCAO. Serial coronal slices of fresh brain tissue were stained with TTC. The average infarct volume was 31.1 mm^3 in wild type mice at 30 h after MCAO, while it was 12.1 mm^3 in *Cirbp*$^{-/-}$ mice and significantly reduced by 61% in comparison with wild type mice (Fig. 2). In addition, there was no significant change in TNF-α protein expression in the brain of *Cirbp*$^{-/-}$ mice after MCAO as determined by Western blotting (at 30 h = 1.11 ± 0.15 and at 48 h = 0.962 ± 0.09 after MCAO when compared to the sham animals as 1).

Allograft inflammatory factor 1 (AIF1), also known as ionized calcium-binding adapter molecule 1, is often used as a microglia activation marker [21]. The numbers of AIF1 immunostaining cells in the brain were markedly increased in wild type mice at 30 h after MCAO (Fig. 3). Furthermore, the shape of microglia changed from the 'resting' status with long branching processes and a small cellular body to the 'active' status with a large amoeboid shape after MCAO (Fig. 3). In contrast, the numbers of AIF1 staining and the activating microglia were reduced in *Cirbp*$^{-/-}$ mice in comparison with wild type mice (Fig. 3). These results suggested that CIRP might play a role in regulating inflammation and the activation of microglia in brain injury with stroke.

3.3. Effect of hypoxia on CIRP expression and translocation in BV2 cells

After observing the change of CIRP expression in ischemic brain, we examined whether CIRP was regulated under hypoxic stresses *in vitro*. Since microglia play a critical role in neuroinflammation, we measured expression levels of CIRP in microglial BV2 cells exposed to hypoxia. The levels of CIRP mRNA, as determined by real-time PCR, increased by 2.9- and 10.5-fold at 20 h and 30 h of hypoxia, respectively, compared to normoxia ($P < 0.05$; Fig. 4A). The intracellular levels of CIRP protein, as determined by Western blotting, increased by 4.6- and 2.3-fold at 20 h and 30 h of hypoxia respectively, compared to normoxia ($P < 0.05$; Fig. 4B). At 30 h of hypoxia, however, the increase of intracellular CIRP protein levels did not correspond to its mRNA levels. To resolve this discrepancy, we collected the cultured medium

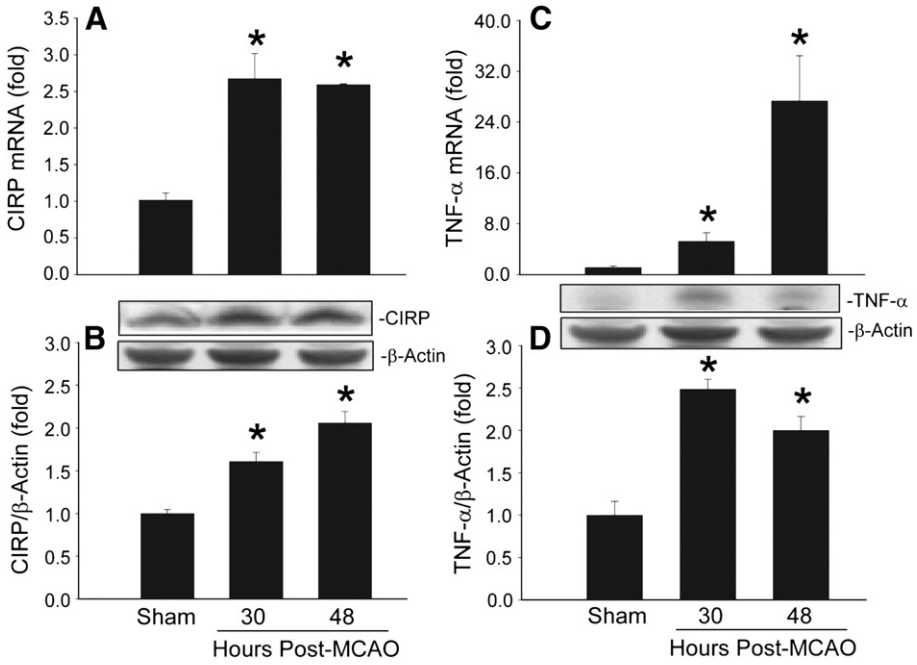

Fig. 1. Induction of CIRP and TNF-α in the mouse brain after MCAO. The brain tissues from wild type mice were harvested at 30 h and 48 h after MCAO. Real-time PCR analysis of gene expression of (A) CIRP and (C) TNF-α. Western blotting measurement of (B) CIRP and (D) TNF-α from lysate of the ischemic portion of the brain. Representative blots of CIRP (19 kDa), TNF-α (26 kDa) and β-actin (43 kDa) are shown. Data are expressed as means ± SE (n = 4/group) and compared by one-way ANOVA and SNK test. *$P < 0.05$ *vs.* sham; sham values were normalized as 1.0.

from BV2 cells grown at normoxic and hypoxic conditions and measured their CIRP protein levels by Western blotting. As shown in Fig. 4C, CIRP protein could be detected in hypoxic cultured media and its levels increased by 7- and 23-fold at 20 h and 30 h of hypoxia respectively, compared to normoxia ($P < 0.05$). Thus, the reduction of intracellular CIRP protein levels at 30 h of hypoxia was most likely due to its release into the cultured medium.

CIRP is a nuclear protein that needs to translocate from the nucleus to the cytoplasm in order to be secreted into the extracellular matrix. To monitor this process, we transfected BV2 cells with a plasmid

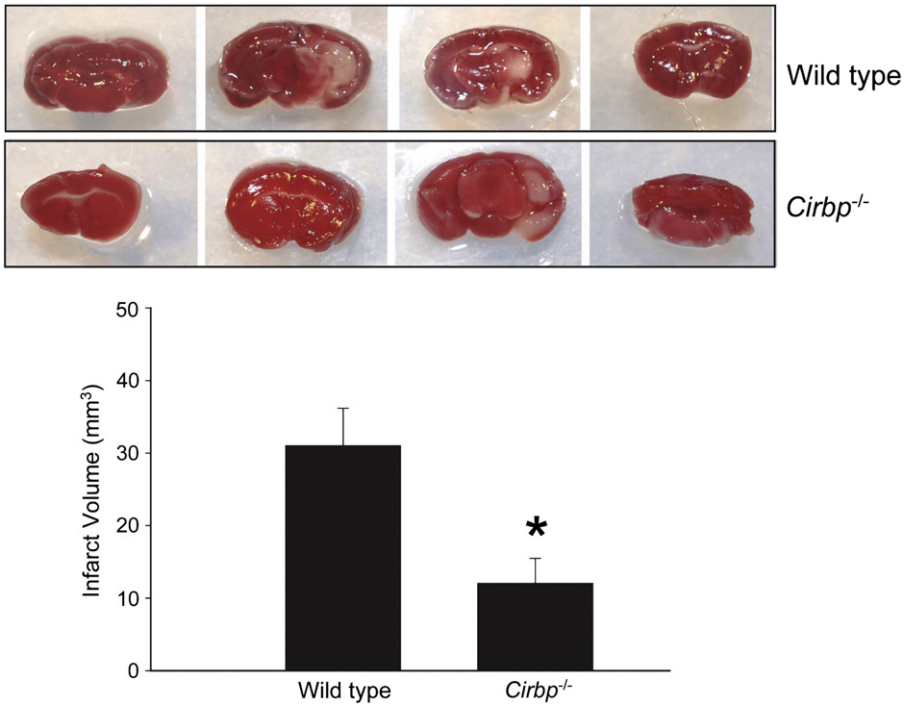

Fig. 2. Effect of CIRP deficiency on infarct volume after MCAO. The brain tissues from wild type and *Cirbp*$^{-/-}$ mice were harvested at 30 h after MCAO. Serial coronal slices of fresh brain tissue were stained with triphenyl tetrazolium chloride. Infarcted area of the brain appears as pale staining on the slice and viable brain area shows plum red color. The representative images are shown. The infarcted area was quantified using NIH ImageJ and infarct volume was calculated. Data are expressed by mean ± SE (n = 6–8/group) and compared by Student's *t*-test. *$P < 0.05$ *vs.* wild type.

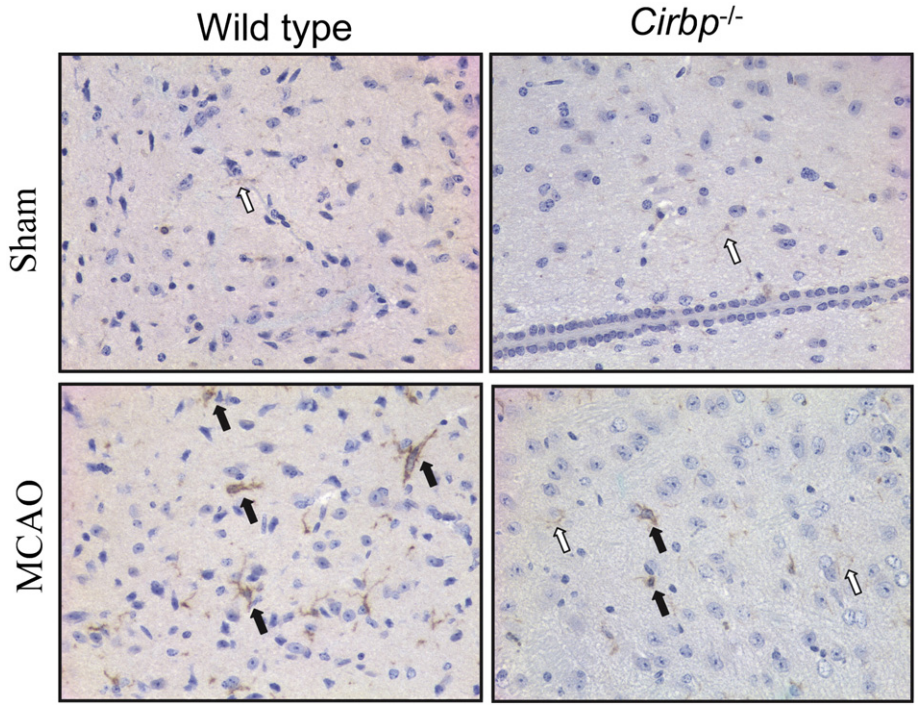

Fig. 3. Effect of CIRP deficiency on activation of microglia after MCAO. The brain tissues from wild type and *Cirbp*$^{-/-}$ mice were harvested at 30 h after MCAO. Brain sections were immunostained with allograft inflammatory factor 1 (AIF1), a microglia activation marker (in brown). Resting microglia are indicated by white arrows and activated microglia are indicated by black arrows. Representative microphotographs of sham and MCAO brain sections are shown. Original magnification, 400×.

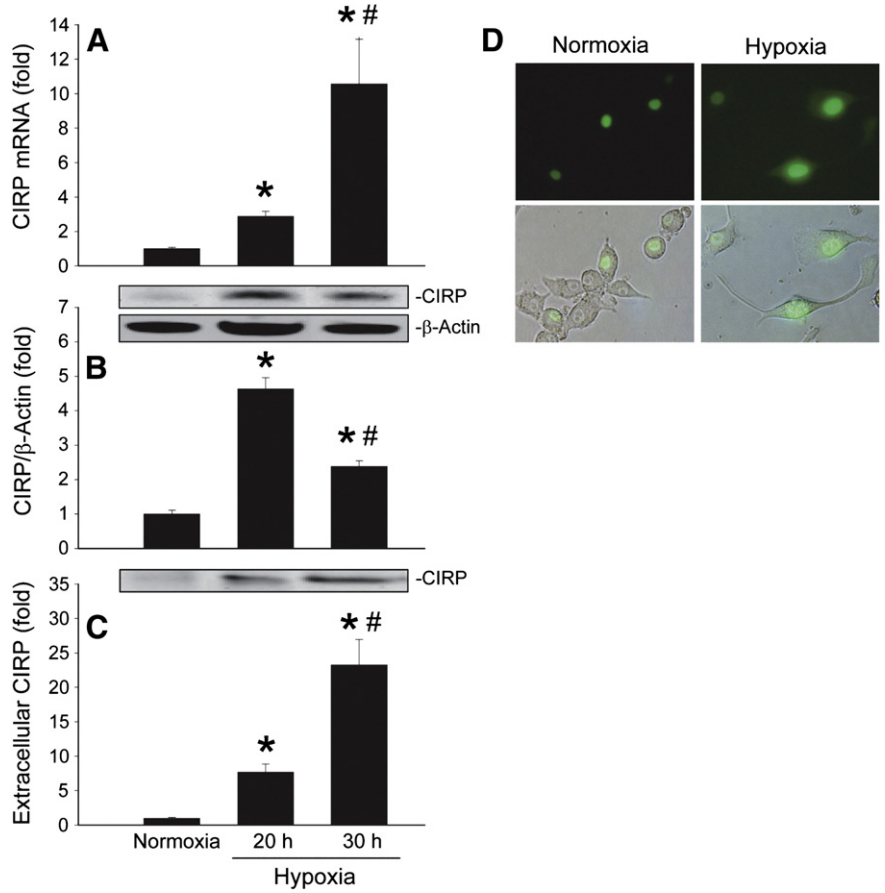

Fig. 4. Induction of CIRP production and translocation in microglia after exposure to hypoxia. Microglial BV2 cells were cultured under normoxia or hypoxia for 20 h or 30 h. (A) Real-time PCR analysis of CIRP gene expression from total cell lysate. Western blotting of CIRP from (B) total cell lysate and (C) cultured conditioned medium. Representative blots of CIRP (19 kDa) and β-actin (43 kDa) are shown. Data are expressed as means ± SE (n = 4–6/group) and compared by one-way ANOVA and SNK test. $^{*}P < 0.05$ *vs.* normoxia; $^{\#}P < 0.05$ *vs.* hypoxia at 20 h. Normoxia values were normalized as 1.0. (D) Images of BV2 cells expressing GFP-CIRP (green) after exposure to normoxia or hypoxia for 24 h. The cells were viewed under fluorescence and light microscope. Original magnification, 400×.

carrying the CIRP gene fused with GFP, followed by exposure to hypoxia. Under a fluorescence microscope, green fluorescence was only observed in the nucleus at normoxia, while it could be detected in both nucleus and cytoplasm after hypoxia (Fig. 4D). This result indicated that hypoxia induced the translocation of CIRP from the nucleus to the cytoplasm in BV2 cells.

3.4. Effect of hypoxia on TNF-α expression in BV2 cells

We further examined hypoxia-induced inflammation by measuring TNF-α expression in BV2 cells. The levels of TNF-α mRNA in BV2 cells increased by 2.2- and 5.1-fold at 20 h and 30 h of hypoxia respectively, compared to normoxia ($P < 0.05$; Fig. 5A). Intracellular protein levels of TNF-α increased by 2.3-fold at 20 h of hypoxia (Fig. 5B). At 30 h of hypoxia, intracellular protein levels of TNF-α were reduced to the levels comparable to those in normoxia (Fig. 5B). In cultured medium, however, TNF-α levels as measured by ELISA increased from 267 pg/ml at normoxia to 516 pg/ml at 30 h of hypoxia (Fig. 5C).

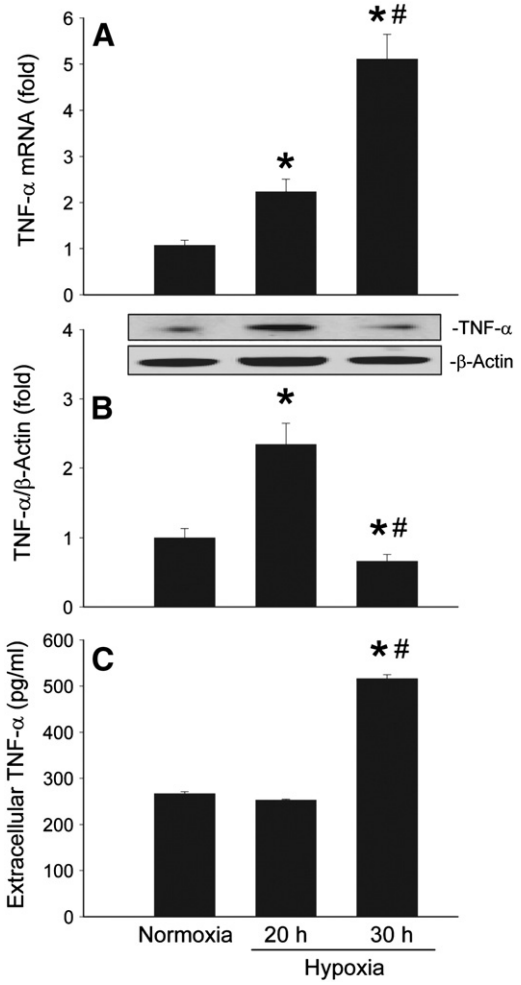

Fig. 5. Induction of TNF-α production in microglia after exposure to hypoxia. Microglial BV2 cells were cultured in normoxia or hypoxia for 20 h or 30 h. (A) Real-time PCR analysis of TNF-α gene expression from total cell lysate. (B) Western blotting of TNF-α from total cell lysate. Representative blots of TNF-α (26 kDa) and β-actin (43 kDa) are shown. (C) The levels of TNF-α in cultured conditioned medium as measured by ELISA. Data are expressed as means ± SE (n = 4–6/group) and compared by one-way ANOVA and SNK test. $^{*}P < 0.05$ *vs.* normoxia; $^{\#}P < 0.05$ *vs.* hypoxia at 20 h. Normoxia values were normalized as 1.0 in A and B.

3.5. Effect of CIRP on TNF-α release and NF-κB activation in BV2 cells

After detecting both CIRP and TNF-α in the cultured medium after hypoxia, we then investigated whether extracellular CIRP could directly regulate TNF-α release. TNF-α levels in the cultured medium of BV2 cells increased from 392 pg/ml to 663 pg/ml after incubating with 1.0 μg/ml of rmCIRP for 20 h (Fig. 6A). To further confirm the specificity of CIRP on stimulating TNF-α release, we co-administered neutralizing antisera to CIRP (10 μg/ml) with rmCIRP (1.0 μg/ml). As shown in Fig. 6A, antisera to CIRP effectively prevented the induction of TNF-α levels by rmCIRP.

NF-κB signaling is the major pathway responsible for upregulating TNF-α expression [22]. To monitor the activation of NF-κB, BV2 cells were transfected with a plasmid containing NF-κB binding elements in the promoter region and a luciferase as reporter gene. Luciferase activities were well detected in the presence of rmCIRP at 1 μg/ml, while it was undetectable in the untreated control (Fig. 6B). The transfected BV2 cells treated with LPS (40 ng/ml) were served as positive control [22] (Fig. 6B). These results indicated that CIRP induced TNF-α release through activating NF-κB pathway in BV2 cells.

3.6. CIRP released from BV2 cells under hypoxia mediates neuron injury

To examine the effect of hypoxia-stressed microglia on neuronal damage, conditioned medium from BV2 cells with or without exposure to hypoxia for 30 h was added to the differentiated SH-SY5Y cells. After 30 h incubation, the caspase activity of SH-SY5Y cells incubated with hypoxic conditioned medium was increased by 1.6-fold in comparison with the cells incubated with normoxic conditioned medium (Fig. 7A). With the addition of antisera to CIRP in the BV2 cell culture, the activation of caspase activity from the hypoxia conditioned medium was

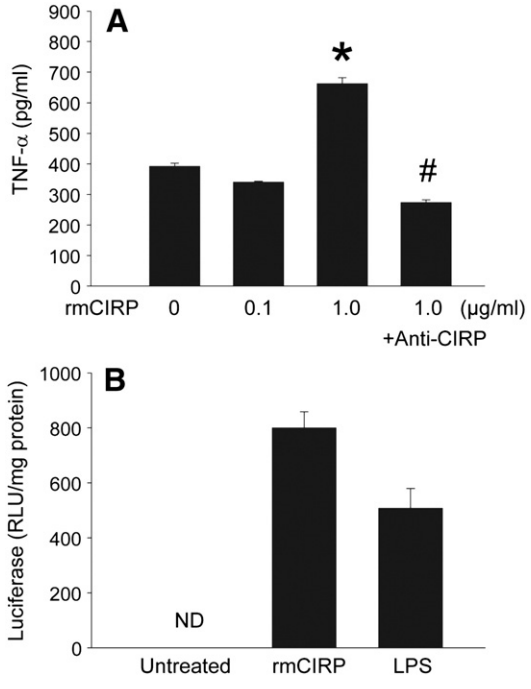

Fig. 6. Effect of CIRP on TNF-α release and NF-κB activation in microglia. (A) TNF-α levels in the cultured medium from BV2 cells incubated with rmCIRP (0, 0.1, or 1.0 μg/ml) or co-administered with neutralizing antisera to CIRP (anti-CIRP, 10 μg/ml) for 20 h, were measured by ELISA. Data are expressed as means ± SE (n = 4) and compared by one-way ANOVA and SNK test. $^{*}P < 0.05$ *vs.* no rmCIRP; $^{\#}P < 0.05$ *vs.* rmCIRP at 1.0 μg/ml alone. (B) The luciferase activity of BV2 cells transfected with a plasmid containing NF-κB binding elements and a luciferase as reporter gene, followed by incubation with rmCIRP (1 μg/ml) or LPS (40 ng/ml) for 20 h. Data are expressed as means ± SE (n = 3). ND, non-detectable; RLU, relative light unit.

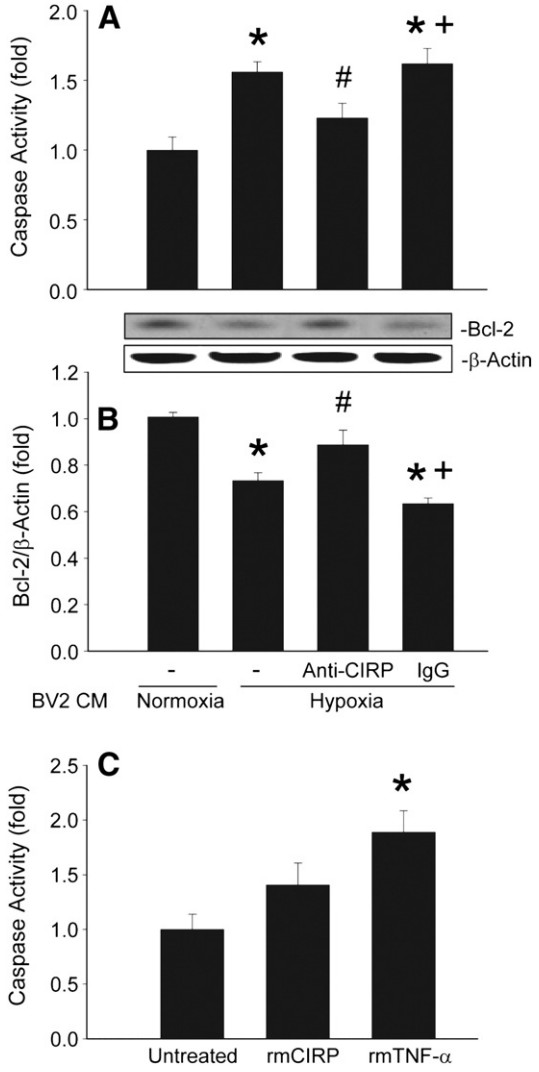

Fig. 7. Effect of the conditioned medium from microglia exposed to hypoxia on neuron injury. The conditioned medium (CM) from BV2 cells exposed to normoxia or hypoxia in the presence of antisera to CIRP (anti-CIRP, 10 μg/ml) or rabbit control IgG (10 μg/ml) for 30 h was added to differentiated SH-SY5Y cells for another 30 h. (A) Caspase activity in SH-SY5Y cells as determined by a fluorimetric assay kit. (B) Western blotting of Bcl-2 protein in SH-SY5Y cells. Representative blots of Bcl-2 (26 kDa) and β-actin (43 kDa) are shown. (C) Caspase activity in differentiated SH-SY5Y cells incubated with rmCIRP (1 μg/ml) or rmTNF-α (5 ng/ml) for 30 h. Data are expressed as means ± SE (n = 4) and compared by one-way ANOVA and SNK test. $^{*}P < 0.05$ *vs.* normoxia or untreated; $^{\#}P < 0.05$ *vs.* hypoxia; $^{+}P < 0.05$ *vs.* hypoxia plus anti-CIRP. Normoxia or untreated values were normalized as 1.0.

inhibited (Fig. 7A). In conjunction, the expression of antiapoptotic Bcl-2 protein in SH-SY5Y cells with hypoxic medium was reduced by 30% in comparison with the cells with normoxic medium (Fig. 7B). With the addition of antisera to CIRP, the expression of Bcl-2 was recovered similarly to that in the cells with normoxic medium (Fig. 7B). The specificity of the effect of antisera to CIRP was validated by comparing with the effect of rabbit control IgG (Fig. 7A & B). In addition, administration of anti-TNF-α antibody (10 μg/ml) to hypoxia conditioned medium also significantly reduced the caspase activation in SH-SY5Y cells, similar to that with the administration of antisera to CIRP (21.9 ± 0.1% *vs.* 21.2 ± 0.1% reduction from the hypoxia conditioned medium), suggesting that the detrimental effect of CIRP on neuronal cells might be mediated by TNF-α.

Since both CIRP and TNF-α could be released from BV2 cells after exposure to hypoxia, we further determined whether these two molecules had a direct effect on the damage of neural cells. As shown in Fig. 7C, the caspase activity of the differentiated SH-SY5Y cells was increased by 41% and 89% with incubation of rmCIRP (1 μg/ml) and rmTNF-α (5 ng/ml) for 30 h, respectively, compared to the untreated control. These results indicated that CIRP and TNF-α could induce apoptosis of neural cells.

4. Discussion

The MCAO procedure performed either permanently or transiently is the most relevant animal model for studying stroke [23]. By using the permanent occlusion model, we have investigated the role of CIRP in responding to an ischemic insult in the brain. Hypoxia/ischemia-elicited inflammatory responses are manifested by the accumulation of proinflammatory cytokines [24–26]. In this study, we first detect the elevation of CIRP gene and protein expression in the mouse brain with stroke which is associated with an increased expression of proinflammatory cytokine TNF-α gene and protein. We further validate the contribution of CIRP in stroke by showing that the infarct volume and TNF-α level of *Cirbp*$^{-/-}$ mouse brain are significantly reduced in comparison with that of wild type mouse brain after MCAO. Thus, expression of CIRP may be a detrimental factor for brain injury during stroke.

Experimental data have shown that ischemia-activated microglia produce a plethora of proinflammatory mediators, including TNF-α, which exacerbate tissue damage [25,26]. It has been reported that microglia are already activated at day 1 after focal cerebral ischemia and remain activated even at days 4 and 7 after stroke [23,27,28]. Although blood-derived macrophages start to be recruited into the ischemic brain tissue after day 3 following stroke, the number of the infiltrating macrophages remains much lower than activated resident microglia [23,27,28]. Consistently, we also observe activated microglia in the brain at 30 h after MCAO by immunohistochemical staining with AIF1. However, activation of microglia was markedly reduced in *Cirbp*$^{-/-}$ mice. Our results suggest that CIRP might play a role in activating microglia for neuroinflammation during brain injury induced by ischemic stroke.

Corresponding to the observation in the animal model, CIRP expression is also upregulated in microglial BV2 cells exposed to hypoxia. Furthermore, CIRP can be released into the extracellular matrix from BV2 cells exposed to hypoxia. Previously, it has been indicated that CIRP can migrate from the nucleus to cytoplasm [29]. By using a molecular biology approach to express CIRP tagged with GFP, here we demonstrate the translocation of CIRP from the nucleus to the cytoplasm in response to hypoxia stimulation. Such release and translocation of CIRP have also been observed in mouse macrophage-like RAW 264.7 cells after exposure to hypoxia in our recent study [14]. Although the detailed mechanisms of CIRP release are still under investigation, lysosomal secretion is one of the pathways for CIRP release under hypoxic stress as demonstrated in RAW 24.7 cells [14].

We also demonstrate an increase of TNF-α gene and protein expression as well as its release in BV2 cells exposed to hypoxia. When comparing the timing of the release, CIRP is detected earlier than TNF-α in the cultured medium (20 h *vs.* 30 h). Furthermore, the addition of rmCIRP can stimulate TNF-α release in BV2 cells, which is blocked by antisera to CIRP. Therefore, extracellular CIRP acts as an upstream regulator of TNF-α release in BV2 cells, which is similar to our previous observation in macrophages [14]. We further identify that extracellular CIRP can activate the NF-κB pathway in BV2 cells by using the NF-κB-luciferase reporter assay. It is well known that activation of NF-κB is the major pathway responsible for the production of various inflammatory mediators, including cytokines, chemokines, and vasoactive peptides [2,4]. Our previous study shows that CIRP has a strong binding to the cell surface receptor TLR4/MD2 complex [14], which can engage in NF-κB activation [30]. We also observe the activation of NF-κB by LPS, a major ligand of the TLR4/MD2 complex [30], indicating the intactness of the TLR4/

MD2–NF-κB signaling pathway in BV2 cells for transmitting extracellular CIRP signal.

Proinflammatory cytokines, especially TNF-α, can lead to neuronal injury or even cell death [24,25]. Several anti-inflammatory approaches have proven to be beneficial in the prevention and treatment of ischemic brain injuries in animal models [31,32]. By using the *in vitro* cell culture system, we detect the induction of apoptosis in neural SH-SY5Y cells by incubation with the conditioned medium of BV2 cells exposed to hypoxia as well as CIRP or TNF-α which are released during hypoxic stresses. With administration of antisera to CIRP as well as to TNF-α in the cultured medium, the induction of apoptosis in SH-SY5Y cells is attenuated. Thus, blocking CIRP activity, such as using anti-CIRP antibodies, may have a therapeutic implication in treating brain injury caused by neuroinflammation in stroke.

CIRP is expressed in the hippocampus and cortex regions of the brain with the same level [8,13]. Several studies have indicated that induction of CIRP expression by moderate hypothermia is associated with the protection of neural cell death from subsequent oxidative stresses [13,33,34]. Thus, intracellular CIRP has been implicated as responsible for hypothermia-induced neuroprotection. Whether mild hypothermia can induce the translocation or release of CIRP has not been studied yet. In contrast, we here demonstrate that extracellular CIRP released from hypoxic stresses acts as an inflammatory mediator to cause neuronal injury. In our recent study, we have characterized CIRP as an endogenous damage-associated molecular pattern molecule (DAMP) or alarmin [14]. DAMPs are released from the host under injury or stress conditions in the absence of infectious pathogens to trigger inflammatory responses [35]. Several other molecules, such as high mobility group box 1 [36], heat shock proteins [37], S100 proteins [38], histones [39], and mitochondrial DNA [40] have also been identified as DAMPs.

In conclusion, we have identified that CIRP, a cold shock protein, serves as a novel mediator in regulating brain inflammation through stimulating TNF-α production in microglia under hypoxic/ischemic stress. The severity of brain damage and inflammation with stroke is ameliorated in CIRP-deficient mice. Administration of neutralizing antisera to CIRP can attenuate TNF-α release from microglia and apoptosis of neural cells exposed to hypoxia medium. These findings provide a new understanding of neuroinflammation and the complex pathogenesis of ischemic stroke. Thus, targeting CIRP may provide a new therapeutic strategy to attenuate brain inflammation and reduce neuron damage in ischemic stroke.

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