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Short communication

Carbon monoxide offers neuroprotection from hippocampal cell damage induced by recurrent febrile seizures through the PERK-activated ER stress pathway

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HIGHLIGHTS

- Plasma CO level raised in a rat model of recurrent febrile seizures (FS).
- Increased hippocampal cell damage and apoptosis in FS rats is attenuated by Hemin.
- Increased *p*-PERK and *p*-eIF2α in FS rats is blocked by ZnPP-IX and enhanced by Hemin.
- CO is neuroprotective in FS-induced hippocampal damage via the PERK-ER stress pathway.

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ABSTRACT

Carbon monoxide (CO) is neuroprotective in various models of brain injury, but the precise mechanisms for this are yet to be established. In the present study, using a rat model of recurrent febrile seizures (FSs), we found an increase in plasma CO, evidence of neuronal damage and apoptosis, an increase in the expression of the endoplasmic reticulum stress (ERS) marker glucose-regulated protein 78 (GRP78) and C/EBP homologous binding protein (CHOP), and an increase in phosphorylated protein kinase RNA-like endoplasmic reticulum kinase (*p*-PERK)/eukaryotic translation initiation factor 2 alpha (*p*-eIF2α) in the hippocampus after 10 FSs. Administration of Hemin (a CO donor) in FS rats alleviated the neuronal damage, reduced neuronal apoptosis, upregulated GRP78 expression, decreased CHOP, and increased *p*-PERK and *p*-eIF2α expression in the hippocampus, compared to FS control rats. In contrast, treating FS rats with ZnPP-IX (a CO synthase inhibitor) aggravated the neuronal damage, enhanced neuronal apoptosis, downregulated GRP78 expression, increased CHOP, and decreased *p*-PERK and *p*-eIF2α expression, compared to FS control rats. These results suggest that endogenous CO limits the neuronal damage induced by recurrent FSs, through the PERK-activated ERS pathway.

1. Introduction

Febrile seizures (FSs) are the most common seizure disorder in children, affecting 2–4% of all children between 6 months and 5 years of age, with a third having more than one episode [1]. Clinical and experimental studies have demonstrated that recurrent FSs may be associated with a number of long-term consequences, such as a decreased seizure threshold, learning difficulties, and memory problems [2].

The hippocampus is the primary brain region involved in the generation of FSs. Importantly, FSs can cause neuronal damage and enhance cytogenesis in the hippocampus [3], which is one of the most common causes of temporal lobe epilepsy. The intracellular pathways underlying seizure-induced neuronal death have not been fully elucidated.

Hippocampal neurons are characterized by a potent endogenous cytoprotective mechanism that involves heme oxygenase (HO) [4]. The cytoprotective capacity of HO is defined by the antioxidant potencies of carbon monoxide (CO) [5,6]. Previously, we provided evidence that CO/HO^{-1} plays an essential neuronal protective role against seizure-induced hippocampal damage in CA_1 and CA_3 in the developing brain [7,8]. However, the mechanisms involved in the cytoprotective effects of CO in the hippocampus during FSs remain unknown. Evidence suggests that endogenous nitric oxide

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(NO) and CO interact with each other and play an important regulatory role in FS-induced hippocampal damage [7]. Recent studies have demonstrated that NO mediates neuronal apoptosis in rats with recurrent FSs, through the endoplasmic reticulum (ER) stress (ERS) pathway [9,10]. Because CO and NO have similar biological properties [11,12], these studies prompted us to test whether CO functions through the ERS pathway in FSs.

Protein kinase RNA-like ER kinase (PERK) is one of the three major ERS sensor proteins, and plays an important role in inducing cellular apoptosis [13]. Normally, PERK is held in an inactive, monomeric state by the binding of the ER chaperone glucose-regulated protein 78 (GRP78). ERS leads to the accumulation of unfolded proteins in the ER lumen. GRP78 dissociates from PERK to bind these accumulated unfolded proteins, leading to PERK activation, phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α), and inhibition of global protein synthesis [14,15]. A study of epilepsy has found that inhibition of eIF2α dephosphorylation significantly reduces ERS and neuronal death induced by kainic acid [16]. However, little is known about the role of the PERK-eIF2α pathway in FSs. In this study, we investigated whether CO protects the hippocampus from the damage induced by recurrent FSs via the ERS pathway; we focused on the activation of the PERK-eIF2α pathway, which is an unfolded protein response branch for cytoprotection.

2. Materials and methods

The animal care and experimental protocols used in this study complied with the Animal Management Rule of the Ministry of Health, China, and the Animal Care Committee of Peking University First Hospital, Beijing, China. Male Sprague–Dawley rats were housed with their mothers under standard laboratory conditions until they were weaned at 21 days. Our studies started with animals aged 21 days and ended with animals aged 40 days. Rats were randomly divided into four groups: control group, FS group, FS + ZnPP-IX group, and FS + Hemin group. The FS model has been previously described in detail [7]. In brief, the control rats were placed into 37 °C water for 5 min, and rats in the other three groups were placed into 45.2 °C water until a seizure occurred. As previously described in detail [17], hyperthermia treatment initiated seizures in rats, which manifested as facial clonus, head nodding, forelimb clonus, rearing (animal in a standing posture aided by the tail and by laterally spreading the hind limbs), and falling back. Water immersion was carried out 10 times, once every 2 days. For rats in the FS + ZnPP-IX group, 45 μmol/kg ZnPP-IX (Sigma, USA) was administered intraperitoneally 30 min before water immersion. For rats in the FS + Hemin group, 40 mg/kg Hemin (Humaike, China) was administered intraperitoneally 30 min before water immersion. An equal volume of normal saline was intraperitoneally injected into rats in the control and FS groups 30 min before water immersion. All the rats were killed 6 h after the final water immersion. Six rats from each group were examined in each experiment, and the controls were age-matched.

Blood samples from each rat were obtained from the abdominal vein and then centrifuged to separate the plasma. Next, 0.5 ml plasma was mixed with 0.5 ml hemoglobin (Hb) solution (derived from a mixture of 0.25 ml fresh-packed rat erythrocytes and 50 ml of 0.25 mol/l ammonia solution). Sodium dithionite (0.1 ml) was then added. The difference in absorbance at 564 and 582 nm of the samples was then read in a double-wavelength spectrophotometer. Both the wavelengths were selected according to the absorbance spectrum of carboxygen–hemoglobin (HbCO) and oxygenated hemoglobin (HbO_2). The difference in absorbance of HbCO was large, and the difference in the absorbance of HbO_2 was zero. The percentage of HbCO was then calculated from a standard curve derived from mixing different proportions of two Hb solutions containing 100% HbO_2 and 100% HbCO. We obtained these solutions by gassing Hb solutions with pure oxygen and CO, respectively. Plasma CO concentration was calculated according to the Chalmers formulae [7,18]:

$$\%\mathrm{HbCO} = \frac{(\Delta \mathrm{O\,D} - 0.033)}{0.0573}$$

$$\frac{\mathrm{CO}\ \mu mol}{1}\ \text{of plasma} = \frac{\mathrm{HbCO}\ (\%) \times \mathrm{Hb(mg/l)} \times 4000}{(64.456 \times 100 \times 0.5)}$$

A part of rats were anesthetized and then perfused through the heart with 0.9% saline followed by 4% paraformaldehyde (PFA) in phosphate–buffered saline (PBS) for 30 min. The brains were carefully removed, post-fixed for 20 h, equilibrated sequentially with 30% sucrose at 4 °C for 72 h, and finally frozen with liquid nitrogen, and stored at −70 °C for further analysis. Brains coronal sections of 10 μm thickness were incubated with HO^{-1} rabbit polyclonal antibody (1:100; Santa Cruz Biotechnology, CA, USA) overnight at 4 °C, and then with a secondary goat anti-rabbit IgG (Zhongshan Goldenbridge Biotechnology, China) at 37 °C for 60 min. Signals were visualized by diaminobenzidine (DAB).

Rat hippocampus were homogenized in lysis buffer (50 mmol/l Tris–HCl, 150 mmol/l NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 10 μg/ml aprotinin, 1 mmol/l phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin, pH 7.5). Hippocampal protein (20 μg per lane) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane and incubated with HO^{-1} rabbit polyclonal antibody (1:1000; Santa Cruz Biotechnology, CA, USA), GRP78 rabbit polyclonal antibody (1:2000; Sigma, St. Louis, MO, USA), CHOP rabbit polyclonal antibody (1:1000 Novus Biological, Littleton, CO, USA), *p*-PERK rabbit polyclonal antibody (1:1000; Santa Cruz Biotechnology, CA, USA) or *p*-eIF2α rabbit polyclonal antibody (1:2000; Cell Signaling Technology, Danvers, MA, USA). Blotted antibodies were visualized by secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2000) and an enhanced chemiluminescence kit. Blots were also probed with β-actin as a loading control. The relative density of the protein bands was measured using an imaging analysis system.

Total RNA in rat tissues was extracted using Trizol reagent and reverse-transcribed by oligo$(dT)_{15}$ primer and M-MLV reverse transcriptase. The reaction mixture for real-time PCR (final volume, 25 μl) was mixed with 2.5 μl of 10 × PCR buffer, 1 μl of 7.5 μmol/l forward and reverse primers, 1 μl of 2.5 mmol/l dNTP mixture, 0.25 μl of Taq DNA polymerase and 2 μl of rat tissue cDNA. PCR products were amplified again using the PCR primers (Grp78 Forward: 5′-TGCCCACCAAGAAGTCTCAGA-3′, Reverse: 5′-TCAAATGTACCCAGAAGGTGATTG-3′, TaqMan probe: 5′-CTTCTCCACAGCTTCTGATAATCAGCCCAC-3′; CHOP Forward: 5′-GGCAGCGACAGAGCCAAA-3′, Reverse: 5′-CAGCTGGACACTGTCTCAAAGG-3′, TaqMan probe: 5′-AACAGCCGGAACCTGAGGAGAGAGAAAC-3′; β-actin Forward: 5′-ACCCGCGAGTACAACCTTCTT-3′, Reverse: 5′-TATCGTCATCCATGGCGAACT-3′, TaqMan probe: 5′-CCTCCGTCGCCGGTCCACAC-3′). The PCR conditions were 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. An extraction of the passaged viruses of all genotypes available was at 10-fold (1×10^{-1} to 1×10^{-6}) dilution and analyzed by both real-time and reverse-transcription PCR. β-Actin in each sample was used to calibrate the amount of sample used for determination.

A part of rats were anesthetized with 4% chloral hydrate (600 mg/kg, intraperitoneally) and perfused through the heart with 0.9% saline followed by 3% PFA and 1% glutaraldehyde in PBS. The brains were removed, and the hippocampi were isolated and cut into approximately 1-mm cubes, after immersion in 3% glutaraldehyde in PBS. The tissues were washed three times in sucrose and