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Research paper

CHOP induces apoptosis by affecting brain iron metabolism in rats with subarachnoid hemorrhage

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ABSTRACT

The endoplasmic reticulum stress-related factor CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) aggravates early brain injury (EBI) in rats after subarachnoid hemorrhage (SAH). Our research aims to investigate the role of CHOP-mediated iron metabolism in EBI after SAH and the underlying mechanism. Male Sprague-Dawley rats were used to establish SAH models. Tunicamycin (Tm) was employed to excite CHOP expression, and two CHOP small interfering RNAs (siRNAs) were used to inhibit CHOP expression. Neurological scores, brain water content, and blood-brain barrier (BBB) permeability were evaluated at 24 h after SAH. Western blotting and immunofluorescence were implemented for the quantification and localization of GRP78 (glucoseregulated protein78), CHOP, C/EBPα (CCAAT/enhancer binding proteinα) and hepcidin. Apoptotic cells were detected by TUNEL staining, and the brain iron content was measured via Perls' staining. The expression of CHOP and hepcidin increased and the expression of C/EBPα decreased after SAH. Knockdown of CHOP decreased the brain water content, reduced Evans blue extravasation, and improved neurological functions. CHOP significantly increased hepcidin levels and significantly decreased C/EBPα levels after SAH. Hepcidin is expressed in the nuclei of neurons and is widely co-localized with TUNEL-positive cells both in the hippocampus and cortex. Along with increased hepcidin expression, the iron content in brain tissue and the apoptosis rate were increased. Thus, CHOP promotes hepcidin expression by regulating C/EBPα activity, which increases the brain iron content, induces apoptosis and is involved in the development of EBI after SAH.

1. Introduction

Subarachnoid hemorrhage (SAH) is a major cause of death and disability (Van Gijn et al., 2007). Early brain injury (EBI) is the most prominent cause of an adverse prognosis for patients with SAH (Caner et al., 2012a). EBI includes a series of pathological changes that occur in brain tissues within 72 h after SAH; high intracranial pressure, autonomic regulation of cerebral blood flow disorders, abnormal cerebral perfusion pressure, blood-brain-barrier destruction, apoptosis, inflammation and oxidative stress are all closely related to EBI (Cahill et al., 2006). Specifically, nervous cell apoptosis plays an extremely important role in the process of EBI after SAH (Hasegawa et al., 2011).

We have previously shown that the expression of CCAAT/enhancer binding protein (C/EBP) homologous protein [CHOP; the key factor downstream of endoplasmic reticulum stress (ERS)] is increased after SAH; when the expression of CHOP was inhibited, the EBI damage was reduced after SAH (He et al., 2012a). However, its specific mechanism is not clear. In recent years, close relations between iron metabolism disorders and EBI severity have been identified. Hepcidin is the core protein involved in iron metabolism because it changes the expression levels of its downstream proteins, such as divalent metal transporter 1 (DMT1), ferroportin 1 (Fpn1) and ceruloplasmin (CP). Hepcidin has been shown to promote DMT1 expression and inhibit FPN1 and CP expression. When DMT1 expression increases, cellular iron uptake is enhanced; when FPN1 and CP expression levels decrease, the intracellular iron output is blocked, leading to a higher intercellular iron content and the induction of apoptosis (Tan et al., 2016). Recent studies have indicated that ERS agonists influence hepcidin expression, and the upstream regulatory sequence of the hepcidin gene combines with CHOP, which suggests that CHOP may regulate hepcidin expression (Vecchi et al., 2009; Park et al., 2001). Because CHOP is the key downstream factor of ERS (Kim et al., 2008; Breckenridge et al., 2003a), two different apoptosis signaling pathways, iron metabolism disorder and ERS, have been mechanistically integrated. However, no

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studies have reported these pathways in SAH.

We used an intravascular puncture method to establish an SAH model and various biological methods to detect changes in the expression of key proteins in the ERS-CHOP-C/EBPα-hepcidin pathway before and after the administration of tunicamycin (Tm) and small interfering RNAs (siRNAs) to clarify whether the ERS-mediated disruption in brain iron metabolism plays an important role in EBI after SAH.

2. Materials and methods

2.1. Experimental animals

Two hundred ninety-nine adult male SD rats housed at headquarters (280–350 g) were obtained from the animal center of Chongqing Medical University (Chongqing, China). Animals were housed in a specific pathogen-free cage in a laboratory with a regular 12/12 h light/dark cycle and controlled temperature and humidity conditions. All experimental programs were approved by the Animal Ethics Committee of Chongqing Medical University. The surgical procedures listed below were performed under deep anesthesia to minimize the animals' suffering, according to the guidelines recommended by Kinetic Research Journal and the Guidelines for Care and Use of Laboratory Animals from the National Institutes of Health. All rats were divided into the different groups shown in Table 1.

2.2. SAH animal model

The currently accepted SAH model that establishes good clinical symptoms is the endovascular perforation method. The SAH animal model via endovascular perforation was performed as previously described (Lee et al., 2009). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally), and a sharpened 4–0 monofilament nylon suture was inserted rostrally into the right internal carotid artery from the external carotid artery stump to perforate the bifurcation of the anterior and middle cerebral arteries. The sham operation group underwent the same procedure as the SAH group, with the exception that the vessel wall was not punctured. The severity of SAH was assessed at euthanasia by investigators who were blinded to the groups as previously described (Sugawara et al., 2008); rats with a score of < 9 were excluded from the study.

2.3. Drug administration

2.3.1. TM injection

TM (Sigma-Aldrich, Inc., St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO, 1 mg/mL) and intraperitoneally injected (4.5 mg/kg) 1 h prior to SAH (Fouillet et al., 2012). The control group received the same dose of DMSO.

2.3.2. CHOP siRNA injection

CHOP siRNA has been reported to significantly suppress CHOP mRNA and protein expression (Prasanthi et al., 2011). Two different CHOP siRNAs were obtained from Guangzhou Ribo Biotechnology Co., Ltd. (Guangzhou, China). The sequence of the first siRNA for CHOP was sense, 5′-GGAAGAACUAGGAAACGGA-3′; and antisense, 5′-UCCGUUU CCUAGUUCUUCC-3′. The second siRNA sequence was sense, 5′-CUGG GAAACAGCGCAUGAA-3′; and antisense, 5′-UUCAUGCGCUGUUUCC CAG-3′ (He et al., 2012b). An irrelevant, scramble RNA served as a control. Briefly, 5 nmol of siRNA in 6 μL of sterile phosphate-buffered saline (PBS) was injected into the right lateral ventricle of each rat through a burr hole using a sterile 10-μL Hamilton syringe at a rate of 0.5 μL/min with the following coordinates: 1.5 mm posterior, 1.0 mm lateral, and 3.2 mm below the horizontal plane of bregma (Suzuki et al., 2010). The sham group also received a burr hole; however, they were not subjected to intracerebroventricular injections. After 10 min, the needle was removed, and the burr hole was carefully plugged with bone

Table 1
Experimental design and number of animals used in each group.

Step 1	Groups	WB	IF	Number that died	Step 2	Groups	Neurobehavioral score	Brain water content	BBB disruption	Number that died	Step 3	Groups	WB	IF	IHC	TUNEL-IF	TUNEL-IHC	Perls' Prussian blue staining	Number that died
	Sham	3	–			Sham	12	12	12			Sham	3	3	3	3	3	3	
	6 h	3	–			SAH	12	12	12			SAH	3	6	3	3	3	3	
	12 h	3	–			SAH + DMSO	6	6	6			SAH + DMSO	3	–	–	–	–	–	
	24 h	3	3			SAH + Tm	6	6	6			SAH + Tm	3	3	3	3	3	3	
	48 h	3	–			SAH + scramble siRNA	6	6	6			SAH + scramble siRNA	3	–	–	–	–	–	
	72 h	3	–			SAH + COHP siRNA	6	6	6			SAH + COHP siRNA	3	3	3	3	3	3	
	Total	18	3	5		Total	42	42	42	28		Total	30	15	12	12	12	12	14

Experimental design and animal group classification. SAH, subarachnoid hemorrhage; DMSO, dimethyl sulfoxide; siRNA, small interfering ribonucleic acid; CHOP, CCAAT/enhancer binding protein homologous protein; WB, western blotting; IF, Immunofluorescence; IHC, immunohistochemistry.