Brain & Development 37 (2015) 747–752

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

www.elsevier.com/locate/braindev

Original article

Cerebral hypoxia–ischemia increases toll-like receptor 2 and 4 expression in the hippocampus of neonatal rats

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Received 9 June 2014; received in revised form 29 November 2014; accepted 1 December 2014

Abstract

Aim of the study: Recent reports provide evidence that Toll-like receptors (TLRs) play a crucial role in cerebral ischemic injuries and neuronal cell death. The precise role of TLRs in mediating neuronal damage remains to be fully elucidated. In this study, we investigated the expression of TLR2 and TLR4 in the hippocampus of the neonatal rats.

Materials and methods: Postnatal day 7 Sprague–Dawley rats were used. The hypoxia–ischemia brain injury models were made by ligature of the left common carotid artery and then inhalation of 8%O_2/92%N_2 for 2 h. TLR2 and TLR4 expression was assessed with immunohistochemical staining and Western blot assays at 3 d, 7 d, and 14 d after injury.

Results: At the 3 d and 7 d time points, we found a significant increase in the number of TLR2 and TLR4 positive cells in the hippocampus of the HI group when compared with the sham group ($P < 0.01$). Western blot showed similar results.

Conclusions: The study indicates that TLR2 and TLR4 are involved in the hypoxic–ischemic injury of full-term neonatal brains.

Keywords: Brain; Hypoxia–ischemia; Neonate; Toll-like receptor 2; Toll-like receptor 4

1. Introduction

Hypoxic–ischemic encephalopathy remains a major cause of neonatal death and long term disabilities [1,2]. Cerebral hypoxia–ischemia (HI) is a condition of complex pathology that includes several inflammatory events. Although there has been tremendous progress in neonatal care, management strategies that minimize the morbidity of hypoxic–ischemic encephalopathy have been limited.

Experimental studies have demonstrated that neonatal HI triggers a broad inflammatory reaction in the brain, including activation of the innate immune system [3]. Toll-like receptors (TLRs) are key components of the innate immune system that recognize a wide variety of pathogen-associated molecular patterns. The TLR family consists of 13 members, and TLR 1–9 are expressed in both mice and humans [4,5]. Recently, accumulating evidence suggests that TLRs are important mediators of brain injury [6]. Therefore, understanding TLRs and their relationship to cerebral disease is becoming increasingly important to basic and clinical scientists.

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http://dx.doi.org/10.1016/j.braindev.2014.12.001

TLR2 and TLR4 are two important components of the brain's innate immune system, and these receptors are mainly expressed in microglia, astrocytes, neurons, and endothelial cells. It was recently shown that TLR2 and TLR4 are pivotal for sterile organ injury, including ischemic brain injury [7,8]. Moreover, TLR2 and TLR4 were found to be important in the pathological progression of cerebral ischemia and reperfusion [9,10]. However, a paucity of studies have investigated the role of TLRs in neonatal ischemic brain injury [11,12].

Here, we examined the expression of TLR2 and TLR4 in the neonatal brain of control animals and after HI injury using a well-established animal model of neonatal brain damage. Because the most common sequelae of brain injury in term newborns is learning and cognitive dysfunction, and the hippocampus is the most important tissue for learning and cognitive function, so we focused the hippocampus in the study.

2. Materials and methods

2.1. Animal model

We obtained 36 postnatal day 7 Sprague–Dawley rats from the Experimental Animal Center at the Fudan University in China. The rats were randomly divided into the HI or sham groups. Animals were anesthetized with anhydrous diethyl ether. The criterion for an acceptable depth of anesthesia included a normal respiratory rhythm and no response to stimuli. As described in our previous study [13], a midline neck incision was made and the left common carotid artery was identified once the rats were fully anesthetized. The artery was separated from the vagus nerve, ligated using two 5–0 silk surgical sutures, and severed between the sutures. The incision was then closed with surgical clips, and the animals were left to recover for 2 h. Rectal temperature was maintained at 37 ± 0.5 °C throughout the procedure using a feedback-regulated water heating system. After recovery, animals were placed in an airtight chamber. The air temperature of the chamber was maintained at 36–36.5 °C, and a constant flow of $8\%O_2/92\%N_2$ hypoxic gas was maintained through the chamber for 2 h. The animals were returned to their home cages after a 1 h recovery period. For rats in the sham group, the left common carotid artery was separated but not ligated, and these animals were not exposed to hypoxic conditions.

All efforts were made to minimize both the suffering and the number of animals used. All procedures were conducted in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Care of Experimental Animals Committee of Fudan University.

2.2. Histology

For each group, 6 rats were euthanized at 3, 7, and 14 days after injury. The rats, used for Hematoxylin–eosin (HE) staining and immunohistochemical staining were transcardially perfused with heparinized saline followed by 4% paraformaldehyde at the above mentioned time points. Next, the brain was removed and immersion-fixed in 4% paraformaldehyde for 24 h at 4 °C. The brain was then dehydrated through graded sucrose (10%, 15%, and 30% for 24 h at each grade) at 4 °C, embedded in OCT embedding medium, and preserved at −80 °C. Frozen brain sections cut at a thickness of 30 μm were used for immunohistochemical staining.

The remaining rats were anesthetized with anhydrous diethyl ether and euthanized at the same time points. Next, the brain was removed and the hippocampus was dissected, rinsed with 0.1 M PBS, and preserved in liquid nitrogen for subsequent Western blot analysis.

2.3. Immunohistochemical staining

For immunohistochemical staining, hippocampal sections were washed (0.1 M Tris, pH 7.6, 15 min), denatured (2 N HCl, 37 °C, 30 min), rinsed (0.1 M PBS, 10 min), incubated with 1% H_2O_2 in 0.1 M Tris for 30 min, rinsed again, blocked (10% normal goat serum, 37 °C, 30 min), and then incubated overnight at 4 °C with a mouse anti-rat monoclonal primary antibody for anti-TLR2 (1:200, Epitomics, USA) or anti-TLR4 (1:200, Santa Cruz, USA). Next, all sections were washed and incubated with an avidin–biotin-peroxidase complex for 1 h at 37 °C. Staining was completed using diaminobenzidine as a chromogen, and then the sections were rinsed, mounted, dried, and cover slipped.

Immunohistochemical staining was visualized using microscopy (Leica, Germany). For each rat, 3 sections were randomly selected for analysis. All counts were performed using a 200× objective lens on a microscope. The total number of positive cells/mm^2 was calculated using Image-Pro Plus 6.0 software.

2.4. Western blot assay

Frozen hippocampal tissue was cut into small pieces and homogenized in 0.5 mL of RIPA buffer (150 mmol/L NaCl, 1% N-40, 0.5% deoxycholate, 0.1% sodium dodecylsulfate, 50 mmol/L TRIS-hydrochloric acid, 2 mmol/L phenylmethylsulfonyl fluoride, pH 7.4), transferred into small tubes and rotated at 4 °C overnight. After centrifugation at 10,000*g* for 30 min, the supernatant was collected and protein concentrations were determined with the Enhanced BCA Protein Assay Kit (Beyotime Biotechnology, Haimen, China).