ELSEVIER

Journal of Neuroimmunology 190 (2007) 101 – 111

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroim

Activation of Toll-like receptor 4 signaling contributes to hippocampal neuronal death following global cerebral ischemia/reperfusion

Fang Hua [a], Jing Ma [a], Tuanzhu Ha [a], Yeling Xia [a], Jim Kelley [b], David L. Williams [a], Race L. Kao [a], I. William Browder [a], John B. Schweitzer [c], John H. Kalbfleisch [d], Chuanfu Li [a,*]

[a] *Department of Surgery, East Tennessee State University, Johnson City, TN 37614, United States*
[b] *Department of Internal Medicine, East Tennessee State University, Johnson City, TN 37614, United States*
[c] *Department of Pathology, East Tennessee State University, Johnson City, TN 37614, United States*
[d] *Department of Biometry and Medical Computing, East Tennessee State University, Johnson City, TN 37614, United States*

Received 15 June 2007; received in revised form 6 August 2007; accepted 27 August 2007

Abstract

Toll-like receptors (TLRs) play a critical role in the induction of innate immune responses which have been implicated in neuronal death induced by global cerebral ischemia/reperfusion (GCI/R). The present study investigated the role and mechanisms-of-action of TLR4 signaling in ischemia-induced hippocampal neuronal death. Neuronal damage, activation of the TLR4 signaling pathway, expression of pro-inflammatory cytokines and activation of the PI3K/Akt signaling pathway in the hippocampal formation (HF) were assessed in wild type (WT) mice and TLR4 knockout ($TLR4^{-/-}$) mice after GCI/R. GCI/R increased expression of TLR4 protein in the hippocampal formation (HF) and other brain structures in WT mice. Phosphorylation of the inhibitor of kappa B (p-IκB) as well as activation of nuclear factor kappa B (NFκB) increased in the HF of WT mice. In contrast, there were lower levels of p-IκB and NFκB binding activity in $TLR4^{-/-}$ mice subjected to GCI/R. Pro-inflammatory cytokine expression was also decreased, while phosphorylation of Akt and GSK3β were increased in the HF of $TLR4^{-/-}$ mice after GCI/R. These changes correlated with decreased neuronal death/apoptosis in $TLR4^{-/-}$ mice following GCI/R. These data suggest that activation of TLR4 signaling contributes to ischemia-induced hippocampal neuronal death. In addition, these data suggest that modulation of TLR4 signaling may attenuate ischemic injury in hippocampal neurons.

Keywords: TLR4; Cerebral; Ischemia; Reperfusion

1. Introduction

Global cerebral ischemia/reperfusion (GCI/R) induces neuronal death, especially in the hippocampal formation (HF) (Nitatori et al., 1995). Recent evidence suggests that innate immune and inflammatory responses play a critical role in ischemia-induced neuronal damage (Lambertsen et al., 2004; Liao et al., 2001). However, the precise molecular mechanisms by which immune/inflammatory responses are involved in ischemia-induced neuronal death are unclear.

Toll-like receptors (TLRs) are a family of signal transduction molecules and play a critical role in the induction of innate and adaptive immunity (Aderem and Ulevitch, 2000). TLR-mediated signaling pathways mainly stimulate the activation of NFκB which is an important nuclear transcription factor for regulating expression of genes involved in innate and inflammatory responses (Hoshino et al., 2002; Porter and Janicke, 1999; Toshchakov et al., 2002). Recent studies have shown that activation of the TLR4-mediated NFκB pathway plays a role in ischemia/reperfusion (I/R) injury. For example, we (Hua et al., 2007; Li et al., 2005; Ha et al., 2006) have reported that TLR4-mediated NFκB signaling contributes to myocardial ischemia/

* Corresponding author. Department of Surgery, East Tennessee State University, Campus Box 70575, Johnson City, TN 37614-0575, United States. Tel.: +1 423 439 6349; fax: +1 423 439 6259.
E-mail address: Li@mail.etsu.edu (C. Li).

0165-5728/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneuroim.2007.08.014

reperfusion injury and TLR4 deficiency protects the myocardium from ischemic injury. TLR4 is also involved in the pathogenesis of I/R injury in liver (Zhai et al., 2004), kidney (Kim et al., 2005) and lung tissues (Shimamoto et al., 2006), TLRs have been identified in the central nervous system (CNS) and are thought to play an important role in the brain's response to pathogens as well as toxic cell debris (Bottcher et al., 2003; Bsibsi et al., 2002; Maslinska et al., 2004) and inflammatory or autoimmune CNS diseases (Chakravarty and Herkenham, 2005; Kerfoot et al., 2004). Recent studies (Cao et al., 2006; Caso et al., 2007) using a permanent and longstanding focal cerebral ischemia model have shown that infarct size is reduced in TLR4-deficient mice compared with wild type (WT) mice. It is still unclear why TLR4 deficiency results in the protection of brain from ischemic injury.

In the present study, we investigated the role and mechanisms-of-action of TLR4 in ischemia-induced hippocampal neuronal death using a murine model of global cerebral ischemia/reperfusion (GCI/R). We observed that neuronal death/apoptosis and the levels of pro-inflammatory cytokine expression in the HF of TLR4-deficient mice were significantly less than in age-matched wild type mice following GCI/R. Importantly, the levels of phosphorylated Akt and GSK3β in the HF of TLR4$^{-/-}$ mice were significantly higher compared with WT type mice after GCI/R. Our data indicates that TLR4-mediated signaling contributes to ischemia-induced hippocampal neuronal death. Our results suggest that modulation of TLR4 may attenuate the inflammatory response and concomitantly enhance activation of the PI3K/Akt pathway, thus protecting hippocampal neurons from ischemia injury.

2. Materials and methods

2.1. Animals

C57BL/10ScCr (TLR4$^{-/-}$) and C57BL/10ScSn (wild type, WT) mice (male, 25–30 g, age: 8–12 weeks) were obtained from Jackson Laboratory and maintained in the Division of Laboratory Animal Resources at East Tennessee State University (ETSU). The experiments outlined in this manuscript conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). All aspects of the animal care and experimental protocols were approved by the ETSU Committee on Animal Care. The mice were divided into four groups: TLR4$^{-/-}$ mice subjected to GCI/R (TLR4$^{-/-}$ I/R, n=11), wild type mice subjected to GCI/R (WT-I/R, n=11), TLR4$^{-/-}$ sham operation (TLR4$^{-/-}$S, n=8) and WT sham operation (WT-S, n=8). Seventy-two hours after GCI/R (n=6/group) or sham operation (n=5/group) mice were anesthetized with Ketamine and transcardially perfused with normal saline followed by 30 ml of 4% buffered paraformaldehyde, pH 7.4. The brains were removed, postfixed, embedded in paraffin and cut into sections (7 μm). In a separate experiment, 6 h after GCI/R (n=5/group) or sham operation (n=3/group), the mice were sacrificed by ketamine overdose and the brains were removed and stored at −80 °C for isolation of cellular proteins.

2.2. Induction of global cerebral ischemia/reperfusion (GCI/R)

Our laboratory established a mouse model of GCI/R, in which cerebral ischemia was induced by occlusion of the common carotid arteries (CCA) bilaterally and the left subclavian artery (LSA) together with right subclavian artery (RSA) stenosis under controlled ventilation (Hua et al., 2006). In brief, anesthesia was induced by 5.0% Isoflurane and was maintained by inhalation of 1.5% Isoflurane driven by 100% oxygen flow using the EZ-Anesthesia system (Euthanex Corp., Palmer, PA). The trachea was intubated and the lungs were mechanically ventilated at a rate of 110 breaths per min with a total delivered volume of 0.5 ml. Body temperature was regulated at 37.0 °C by surface water heating. Under a surgical microscope, stenosis of the RSA was produced as previously described (Hua et al., 2006). Then the left CCA, the LSA and the right CCA were gently isolated and clamped with microsurgical clamps, respectively. Cerebral ischemia was maintained for 12 min and reperfusion started when the clamps were removed. The ischemic condition and the sufficiency of reperfusion were confirmed by regional cerebral blood flow (rCBF) detected by the PeriFlux system 5000 (Type PE5001, Jarfalla, Sweden). The chest was closed in layers and all of the incisions were sutured. The mice were placed in a cage kept at 31 °C for the following 3 h and then returned to the animal care room.

2.3. Evaluation of neuronal damage in the hippocampal formation (HF)

Brain sections were stained with 0.1% cresyl violet according to Nissl's method (Lee et al., 2004). Briefly, brain tissues from each group were embedded in paraffin and cut at 7 μm. Paraffin sections were deparaffinized and brought to water, then stained with 0.1% cresyl violet for 2 min. Slides were washed in running tap water for 2–5 min, dehydrated, cleared and then mounted with coverslips. Viable neurons were defined as neurons in which the nucleus appeared normal (a round nucleus showing clear nucleoplasm with a nucleolus). Neurons irreversibly damaged by ischemia exhibit pyknosis and/or nuclear fragments and shrunken cell bodies. Representative sections from the level described in the next paragraph were analyzed for neuronal damage in the hippocampal formation (HF). The HF is formed by two cortical laminae embedded into each other: the cornu ammonis (CA) and the gyrus dentatus (DG). The CA area can be further subdivided into CA1, CA2, CA3, and CA4. The CA1 area is the region most vulnerable to ischemia. A 0–4 neuropathological score (NPS) was used as previously described (Lee et al., 2004): grade 0, no damage to any HF fields; grade 1, scattered ischemic neurons in CA1 area; grade 2, moderate ischemic damage in field CA1 (less than half of pyramidal cells affected); grade 3, severe damage to pyramidal cells in field CA1 (more than half of pyramidal cells affected); grade 4, extensive cell damage in all HF fields.

2.4. Immunohistochemistry (IHC) staining

The expression of TLR4, phosphorylation of NFκB-p65 (p-NFκB-p65) and cleaved caspase-3 activity were examined by IHC as described previously (Hua et al., 2005). The primary antibodies