Neurochemistry International 54 (2009) 403–409

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Apolipoprotein E-deficient mice are more vulnerable to ER stress after transient forebrain ischemia

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ARTICLE INFO

Article history:
Received 6 November 2008
Received in revised form 15 January 2009
Accepted 19 January 2009
Available online 6 February 2009

Keywords:
apoE$^{-/-}$ mice
Forebrain ischemia
ER stress
Hippocampus

ABSTRACT

Apolipoprotein E-deficient (apoE$^{-/-}$) mice have been shown to have increased vulnerability to neuronal damage induced by cerebral ischemia; however, the mechanism of this increased vulnerability remains unclear. In order to define the role of the apoE protein against ischemia-induced ER stress and cell death, experiments were performed to compare ER stress-associated chaperones and signal proteins in the hippocampus of apoE$^{-/-}$ mice to those of WT mice after being subjected to forebrain ischemia and reperfusion. Although neuronal loss in area CA1–CA3 of the hippocampus was observed 3 days after ischemia in both types of mice, the damage in apoE$^{-/-}$ mice was more severe. In apoE$^{-/-}$ mice, a more extensive increase in 78-kDa glucose-regulated protein (GRP78) was observed after the insult, whereas the level of GRP94 was not changed. The expression of both C/EBP homologous protein (CHOP) and caspase-12 was increased in the hippocampus in both WT and apoE$^{-/-}$ mice after ischemia. The increased levels of CHOP in apoE$^{-/-}$ mice were significantly higher than those in WT mice, whereas the levels of caspase-12 in the two were comparable. Furthermore, whereas the levels of c-Jun N-terminal kinase (JNK), p-JNK1 and p-JNK2 in WT mice were unchanged after ischemia, they were significantly increased in apoE$^{-/-}$ mice 24 h and 48 h after ischemia. These results suggest that increased vulnerability of the hippocampus to forebrain ischemia and reperfusion in apoE$^{-/-}$ mice is at least partly attributable to perturbed induction of an ER chaperone, GRP 94, and enhancement of the CHOP- and JNK-dependent apoptotic pathway in the hippocampus.

1. Introduction

Apoptosis plays a pivotal role in neuronal cell death resulting from ischemic stroke. Several lines of evidence suggest that apoptosis is regulated by multiple pathways. The two most well-studied pathways are the cell surface death receptor pathway and the mitochondria-initiated pathway (Budihardjo et al., 1999). Recently, another apoptosis-regulatory pathway involved in ER stress has been receiving attention. The condition in which ER function is impaired, so-called ER stress, can lead to an accumulation of unfolded or malfolded proteins in the ER lumen (Kaufman, 1999). In the case of mild ER stress, cells develop a self-protective signal transduction pathway termed the unfolded protein response (UPR), which includes the induction of molecular chaperones in the ER, translational attenuation, and enhancement of ER-associated degradation (Cudna and Dickson, 2003), thus relieving cells from the stress. However, if the damage is too severe to repair, the UPR ultimately initiates the apoptotic pathway (Oyadomari and Mori, 2004; Cudna and Dickson, 2003). Several proteins have been implicated in this apoptotic pathway, including a transcription factor, C/EBP homologous protein (CHOP) (Wang et al., 1996), and the ER-resident caspase, caspase-12 (Nakagawa et al., 2000). In humans, ER stress has been shown to be involved in not only ischemia but also some neuronal diseases, such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis and prion-related disorders (LaFerla, 2002; Ryu et al., 2002; Imai et al., 2001; Nishitoh et al., 2008; Hetz et al., 2003).

Recent evidence indicates that apolipoprotein E (apoE) plays a central role in CNS injury, ALS–parkinsonism dementia complex (Wilson and Shaw, 2007) and the interactions of herpes simplex products (Carter, 2008). Following brain injury, the synthesis of apoE is markedly increased, and it is secreted, predominantly from astrocytes, into the extracellular space (Poirier, 1994). The expression of apoE increased in the selectively vulnerable hippocampus following transient forebrain ischemia (Hall et al., 1995). Neuronal apoE immunoreactivity was significantly increased in all hippocampal areas (CA1, CA2, CA3/4, dentate fascia) of the human brain following global ischemia (Horsburgh et al., 1999a). It is thought that intraneuronal apoE plays a pivotal role in a protective response following injury by providing lipids for neuronal repair and remodeling. Insight into the role of apoE in brain injury has been provided by the development of genetically

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0197-0186/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2009.01.010

modified mice. It has been shown that apoE$^{-/-}$ mice have a poorer outcome in a focal model of ischemia (Laskowitz et al., 1997), and that this response may be isoform-specific (Sheng et al., 1998). These findings support the suggestion that apoE has a protective effect against ischemic neuronal damage; however, the molecular mechanism underlying the apoE-mediated protection against ischemic neuronal damage remains unclear. Brain ischemia in mice has been shown to cause ER-stress-mediated apoptosis of cells in the striatum and hippocampus, and CHOP plays a crucial role in this cell death (Tajiri et al., 2004). More recent studies have demonstrated that markers of ER stress and UPR activation are induced dramatically in macrophages at all stages of atherosclerosis development in apoE$^{-/-}$ mice (Zhou et al., 2005). Taken together, these findings suggest that apoE protects neurons against ischemic cell death by attenuating ER stress during the injury.

In order to define the role of the apoE protein against ischemia-induced ER stress and cell death in the hippocampus, experiments were performed to compare ER stress-associated signal proteins including ER chaperones, GRP 78 and 94, CHOP, caspase-12 and c-Jun N-terminal kinase (JNK) activity in the hippocampus in apoE$^{-/-}$ mice and wild type (WT) mice after being subjected to forebrain ischemia and reperfusion. We found that the increased vulnerability of hippocampal neurons to ischemic damage was at least partly attributable to disturbed induction of ER chaperones and higher UPR activation.

2. Experimental procedures

2.1. Animals

All experimental procedures were approved by the Institutional Animal Center Use Committee of Graduate School of Pharmacy, Nihon University. ApoE$^{-/-}$ mice, originally produced by Zhang et al. (1992), were purchased from the Jackson Laboratory (Bar Harbor, ME) and back-crossed to WT C57BL/6 mice (Charles River Inc., Yokohama, Japan) to have genetic backgrounds of the homozygote. Both mice were bred in this study. Animals were fed standard laboratory chow and given free access to water prior to surgery. After mating of heterozygote, we selected the homozygous and WT mice by polymerase chain reaction (PCR) amplification of genomic DNA extracted from tails. Male mice (10–12 week old) were used in the present study.

2.2. Transient forebrain ischemia

Transient forebrain ischemia was created by bilateral common carotid artery (BCCA) occlusion as described previously (Kitagawa et al., 1998b). Surgical procedures were performed under chloral hydrate anesthesia (450 mg/kg, i.p.) and the rectal temperature was kept at 37 ± 1.5 °C with a heating blanket (Animal Blanket Controller, Nihon Kohden, Tokyo, Japan). Measurement of cortical perfusion was performed by Omega flow meter (OMEGAWAVE, INC, Tokyo, Japan). In order to measure cortical perfusion by laser Doppler blood-flowmetry, a polyacrylamide column for measurement was attached to the intact skull with dental cement, 3.5 mm lateral bregma. For procedures involving BCCA occlusion, the arteries were approached through a midline cervical incision. Both arteries were identified and ligated with small arterial clips for 20 min, after which time the clips were removed, the return of these arteries blood flow was visually confirmed, the incision was closed, and reperfusion was continued for the indicated time. Sham-operated mice received similar operative procedures; however, the arteries, after isolation, were not occluded and incision was closed. All the mice were killed with a lethal dose of the sodium pentobarbital before recovery of tissues.

2.3. Histological assessment and immunohistochemistry

Anesthetized animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and post-fixed for 24 h in the same fixative. Post-fixed brains were embedded in paraffin and sliced on a microtome at a 6 μm thickness. The sections between 1.5 mm and 2.5 mm posterior of the bregma were used for this study. For histological assessment of damage to the hippocampus, the paraffin-embedded brain sections were stained with hematoxylin and eosin (H&E). For semiquantitative evaluation, the degree of damage was assessed in the CA1–CA3 area by percentage of damaged cells: grade 0, no cell damage visible; grade 1, <50% of cells damaged; grade 2, >50% of cells damaged. The length of the CA1–CA3 area with each degree of damage was measured, and the mean histological score was calculated as described previously (Kitagawa et al., 1998a), by the following formula: (1× length with grade 1 + 2× length with grade 2)/(total length from the CA1 to the CA3 area). For single-stranded DNA (ssDNA) staining that indicated apoptotic cells, paraffin sections were processed immunohistochemically. Briefly, the sections were pretreated with 0.1% trypsin in phosphate buffered saline (PBS) for 20 min at 37 °C, incubated with 0.3% H_2O_2 in methanol for 15 min, appropriate 1.5% blocking normal goat serum for 1 h at room temperature and exposed to the anti-ssDNA (DAKO, Japan) antibodies (dilution 1:500) for 24 h at 4 °C. They were then incubated in the biotin-conjugated IgG (Vector Laboratories, Burlingame, CA) against the host of the primary antibody for 1 h and then incubated with avidin–biotin–peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories) for 60 min. Then the immunoreactivities were detected with 0.025% 3,3′-diaminobenzidine (DAB) and 0.075% H_2O_2 in Tris–HCl (pH 7.6). Negative controls were prepared identically expect for omission of the primary antibody. For cell-counting procedure, the number of morphologically damaged ssDNA positive neurons and viable neurons in hippocampal CA1–CA3 regions were manually counted in four brain slices in each animal and the percentage of ssDNA positive neurons was calculated. The results in each group were presented as the mean ± S.E.M.

2.4. Western blotting

Following transient forebrain ischemia, the mice were sacrificed under the sodium pentobarbital anesthesia at 24 h and 48 h after reperfusion. Sham-operated animals served as controls. Brains were rapidly microdissected on an ice-chilled plate. The hippocampus was homogenized using homogenizer (Homogenizer, Subsonic, IWAKI GLASS CO., LTD) in 800 μL buffer containing 10 mM HEPES-NaOH (pH 7.9), 10 mM potassium chloride, 0.1 mM EDTA, 0.1 mM EGTA, 1 M phenylmethane sulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 62.5 μL/mL protease inhibitor cocktail, phosphatase inhibitor cocktail and 62.5 μL/mL 10% Nonidet P-40, centrifuged, and then supernatants were used. Protein concentrations were determined using the method of (Bradford, 1976). Protein extracts were mixed 1:3 in 4× sample buffer containing 125 mM Tris–HCl (pH 6.0), 3% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol and 0.2% Bromophenol Blue. Aliquots from each sample (10 μg protein/lane) were separated by 7.5–12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) paper (Millipore, MA, USA) (100 V, 90 min). The membranes were blocked in blocking buffer containing 20 mM Tris–HCl (pH 7.6), 137 mM NaCl, 5% skim milk for 1 h at room temperature and then treated with anti-caspase-12 (SIGMA, USA) antibody (diluted 1:1000), anti-CHOP (Santa Cruz Biotechnology, USA) antibody (diluted 1:1000), anti-KDEL (StressGen, Canada) antibody (diluted 1:2000) or JNK antibody (diluted 1:1000), Phospho-JNK (Cell Signaling Technology, Boston, MA, USA) antibody (diluted 1:1000) overnight at 4 °C. The membranes were washed repeatedly in TTBS containing 20 mM Tris–HCl (pH 7.6), 137 mM NaCl, 0.05% Tween 20, then HRP-conjugated secondary antibody (diluted 1:20,000) was added for 1 h. Immunoreactive bands were detected by electrochemiluminescence (ECL, Amersham Pharmacia Biotech). Optical density on the blots was measured with Scion imaging software (www.scioncorp.com).

2.5. Immunofluoresence

Post-fixed brains were immersed for 24 h in PBS containing 30% sucrose, and coronally sectioned on a cryostat at an 18 μm thickness. After blocking nonspecific binding with 1.5% normal goat serum, the sections were incubated anti-CHOP antibody (diluted 1:200) for 24 h at 4 °C. After washing with PBS, the sections were incubated for 1 h with Alexa Fluor 488-conjugated goat IgG (diluted 1:500, Molecular Probes, USA). After rinsing with PBS, the sections were analyzed using a confocal laser microscope (Zeiss LSM-410, Germany). Negative controls were prepared by omitting the primary antibody.

2.6. Statistics

Statistical significance was assessed by a paired Student's *t*-test when 2 groups were compared, and one-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple test when multiple groups were compared.

3. Results

3.1. Hippocampal neurons in apoE$^{-/-}$ mice are more vulnerable to ischemia/reperfusion injury than those in WT mice.

There were no significant differences in any of the parameters of cerebral blood flow and rectal temperature between the WT and apoE$^{-/-}$ mice during and after transient forebrain ischemia (Table 1).

Delayed neuronal death in the hippocampus was compared between WT and apoE$^{-/-}$ mice. Histological evaluation of hippocampal neurons by hematoxylin and eosin staining (Fig. 1) and ssDNA staining (Fig. 2) revealed that neurons in the CA regions