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PBN fails to suppress in delayed neuronal death of hippocampal CA1 injury following transient forebrain ischemia in gerbils

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ABSTRACT

Free radicals have been suggested to be involved in the genesis of ischemic brain damage, as shown by the protective effects of alpha-phenyl-*N*-tert-butyl nitrone (PBN), a spin trapping agent, in ischemic cerebral injury. However, the involvement of free radicals in transient ischemic-induced delayed neuronal death is not fully understood. To clarify this, in the present study, we evaluated the effect of PBN on delayed neuronal death and on the levels of free radicals in hippocampal CA1 region in the gerbil. The administration of PBN (10 mg/kg, i.v.) failed to show any preventive effect on the delayed neuronal death, examined by hematoxylin and eosin staining and the TUNEL method. Furthermore, we observed no free radical formation in delayed neuronal death, determined immunohistochemically using a specific 8-OHdG antibody, after transient ischemic insult. These results suggest that free radical formation may not contribute to the formation of delayed neuronal death.

1. Introduction

It has been suggested that free radicals contribute to the neuronal injury occurring during trauma, oxygen toxicity, Parkinson's disease, aging, and ischemia [8,36,37]. There is accumulating evidence that oxygen free radicals are responsible for the neuronal damage in the course of brain ischemia [4,22,34]. We also have reported that specific free radical scavengers reduced neuronal degeneration in a transient focal cerebral ischemia model [24].

Ischemia-reperfusion perturbs the oxygen supply and energy metabolism of the brain, causing oxidative stress by generating free radicals [7]. Free radicals cause damage to proteins, lipids, and nucleic acids [9]. Interestingly, Lin et al. reported that 8-hydroxy-2′-deoxyguanosine (8-OHdG) is a specific marker of free radical induced neurodegeneration after forebrain ischemia-reperfusion in mouse stroke model [21]. Miwa et al. also reported that 8-OHdG monoclonal antibody (N45.1) is a useful tool as the marker of degeneration of cardiomyocytes after myocardial infarction [23].

Transient cerebral ischemia initiates a process of cellular events that leads to delayed neuronal degeneration of several brain regions both in humans and in animal models [16,30–32]. A brief episode of ischemia causes "delayed neuronal death" in the CA1 sector of the hippocampus [19,33]. Death of the CA1 pyramidal neurons following forebrain ischemia causes a variety of neurological dysfunctions such as depression and memory deficits [2,27]. Of note, the post-ischemic DNA fragmentation in the hippocampal CA1 area in experimental ischemic models is a key component of delayed neuronal death, and is considered as a typical apoptosis process [15–17].

Alpha-phenyl-tert-butyl-nitrone (PBN), a spin trapping agent, is believed to have protective actions in ischemia-reperfusion injury of brain by forming adducts of oxygen free radicals including OH radical [34]. Cao and Phillis have demonstrated that PBN significantly reduces cerebral infarction and decreases neurological deficit after ischemia using a rat model of persistent occlusion of the middle cerebral artery (MCA) [5]. PBN also significantly reduces cerebral infarction after transient occlusion of MCA in rats [20,24,38].

In the transient forebrain ischemia model, PBN reduces mortality [10] and neuronal damage in the CA1 area of hippocampus [6] after ischemia. On the other hand, another antioxidant like PBN, dimethylthiourea, failed to prevent post-ischemic CA1 damage in the rat [28]. Thus, the effects of PBN against delayed neuronal death are contradictory or species-dependent. Furthermore, we have shown that radical scavengers failed to reduce the neuronal injury following transient forebrain ischemia in gerbils [35]. Interestingly, it has been reported that oxidative damage contributes to neuronal injury in transient forebrain ischemia in gerbils [1]. To clarify the contribution of free radicals in delayed neuronal death in gerbils, we determined the effect of PBN on delayed neuronal

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death following transient ischemic insult, and we also evaluated the contribution of free radicals in delayed neuronal death in gerbils by immunohistochemical measurement of free radicals using a specific 8-OHdG antibody.

2. Materials and methods

Male Mongolian gerbils, weighing 65–75 g, were subjected to severe forebrain ischemia as described previously [14,25]. Briefly, the bilateral common carotid arteries were isolated through an anterior midcervical incision and occluded with microclips. After 5 min of forebrain ischemia, the clips were removed. Rectal temperature was maintained at 37 ± 0.3 °C using a heating pad, from the induction of anesthesia until 3 h following ischemia. Sham-operated animals underwent the same surgical manipulation, but without occlusion of bilateral common carotid arteries.

To evaluate the effects of PBN on delayed neuronal death, PBN (Sigma, 10 mg/kg, i.v.) or vehicle control (0.9% saline, 0.1 ml/10 g, iv.) was administrated as bolus ten minutes before ischemic insult. Five animals per group were used for each experiment.

After ischemic insult, animals were anesthetized with pentobarbital and perfused transcardially with saline, followed by phosphate-buffered 10% formalin. Brains were removed and processed for paraffin embedding. Coronal sections (3 μm) were cut at the level of the dorsal hippocampus and then used for hematoxylin and eosin (HE) immunohistochemical staining and TUNEL method.

The animals were housed on 12 h light/dark cycles at 22 °C, and were given free access to food and water. We fully complied with the 'Guidelines Concerning Experimental Animals' issued by the Japanese Association for Laboratory Animal Science and exercised due consideration so as not to cause any ethical problem.

2.1. Immunohistochemistry

Anti-8-OHdG monoclonal antibody (N45.1) was purchased from Japan Institute for the Control of Aging: JaICA, (Japan). The deparaffinized sections were blocked to endogenous peroxidase activity by incubation in distilled water containing 3% hydrogen peroxide for 5 min. Antigen retrieval was performed using a 0.01 M citrate buffer (pH6.0) for anti-8-OHdG antibodies by the Pascal® heat-induced target retrieval system (DAKO). Mouse-to-Mouse HRP staining system (ScyTek Laboratories, Inc., Utah, USA) was used to reduce non-specific background staining.

Anti-8-OHdG antibodies, used at a dilution of 1:500 in 2% BSA/PBS, were added on the slides and incubated overnight at 4 °C. 8-OHdG was detected with an UltraTek kit (ScyTek Laboratories) following the manufacturer's instructions. The peroxidase binding sites were detected by staining with 3,3′-diaminobenzidine (DAB) in 50 mM Tris-EDTA buffer, pH7.6. Finally, counterstaining was performed using Mayer's hematoxilin.

2.2. TUNEL staining

TUNEL method was performed as described previously [13] with some modifications to the method by Gavrieli et al. [12]. After incubation with 20 μg/ml proteinase K (Sigma–Aldrich, Tokyo, Japan), the serial sections used for HE staining were immersed in terminal deoxynucleotidyl transferase (TdT; Roche Diagnostics, Japan) buffer (30 nM Trizma base, pH7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). TdT (Roche Diagnostics, Japan) and biotinylated dUTP (Boehringer, Mannheim) were diluted in TdT buffer at a concentration of 0.15 e.u./μl and 0.8 nmol/μl, respectively. The solution was placed on the sections and then incubated at 37 °C for 60 min. The section were covered with streptavidin peroxidase (DAKO) and stained with DAB as a substrate for the peroxidase. Finally, counterstaining was done using Mayer's hematoxylin.

Table 1
Time-course of the morphological damage, immunohistochemistry for 8-OHdG and nuclear DNA fragmentation in CA1 of gerbil brain following 5 min transient forebrain ischemia.

Time-course	HE	8-OHdG	TUNEL
Sham	–	–	–
24 h	–	–	–
48 h	±	–	–
72 h	++	–	++
96 h	++	–	++
2 w	++	–	–

In HE staining, the damage of hippocampal CA1 neurons in each time point was evaluated as four grades according to the percentage of damaged cells as follows; negative, – (0%); weakly positive, ± (1–5%); positive, + (5–50%); strongly positive, ++ (50–100%). The level of nuclear DNA fragmentation, and the immunostaining intensity of 8-OHdG of hippocampal CA1 was evaluated as four grades, negative (–), weakly positive (±), positive (+) and strongly positive (++).

3. Results

As shown in Fig. 1-A, in sham-operated animals, the hippocampal CA1 regions had a compact cell layer of the pyramidal neurons with large round cell nuclei, and showed no TUNEL staining in any cells. In the ischemic control group (vehicle administration), the strong positive reaction indicating DNA fragmentation occurred exclusively in CA1 neurons, which appeared as pyknotic cells in sections stained with HE at 96 h after transient ischemia. Administration of PBN (10 mg/kg, iv.) failed to protect this neuronal degeneration observed in ischemic control group, evaluated by TUNEL method and HE staining (Fig. 1-A and B).

To determine the involvement of free radicals in delayed neuronal death, we evaluated the expression of 8-OHdG over time after transient forebrain ischemia (Fig. 2), together with morphological damage of neurons evaluated using HE staining and DNA damage detected by TUNEL staining (Fig. 2 & Table 1). 8-OHdG expression was not observed at any period after transient ischemia in CA1 region in gerbil hippocampus. At 48 h following transient ischemia, although there were some minor changes in the CA1 neurons, they still had oval nuclei and prominent nucleoli. By TUNEL staining, no cells in the CA1 region were labeled at 48 h. After 72 h, many neurons were eosinophilic and pyknotic. There was a histologic pattern characterized by an increasing number of damaged neurons in the CA1 region. Many neurons in the CA1 region were TUNEL-positive at 72 h, and at 96 h, almost of all neurons in CA1 sector were damaged, with TUNEL-positive cells still present. After 2 weeks, only a few surviving neurons remained in the CA1 region, and TUNEL-positive neurons were not observed.

4. Discussion

We investigated the involvement of free radicals in the transient ischemia-induced delayed neuronal death in hippocampal CA1 region of Mongolian gerbils. The administration of PBN failed to have any preventive effect on the delayed neuronal death, as determined by HE staining and the TUNEL method. Furthermore, we did not observe any free radical formation, as measured by the specific antibody for 8-OHdG, after transient ischemic insult. These results suggest that free radical formation might not contribute to delayed neuronal death.

It has been reported that PBN is neuroprotective in an experimental stroke model [5,20,38], and we have also reported that PBN administration (both 10 mg/kg, i.v. and 100 mg/kg, i.p.) significantly reduces the infarct volume in transient MCA occlusion-induced