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Original article

Activation of acetylcholine receptors and microglia in hypoxic-ischemic brain damage in newborn rats

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Abstract

Objective: We previously showed that acetylcholine receptor (AChR) agonist reduced hypoxic-ischemic brain damage in the newborn rats. To further investigated the interaction between hypoxia and chorinergic anti-inflammatory pathway, we examined the effect of AChR antagonist on brain damage and to see the relation between microglial activation and protective effect of AChR agonist. *Study design:* Seven-day-old Wistar rats were divided into 2 groups, one receiving AChR antagonists to see if they have deleterious effects on hypoxic-ischemic brain damage, and the other receiving AChR agonist, carbachol, to investigate the emergence of microglia in the hippocampus. Rats were subjected to left carotid artery ligation followed by 8% hypoxia. Brains were analyzed histologically and immunohistochemically. *Results:* Antagonists of AChRs significantly enhanced brain damage in 1-h hypoxia-ischemia. In particular, the nicotinic AChR antagonist showed a marked enhancement of brain damage compared to the saline controls ($p < 0.01$). The hippocampal CA1 was most vulnerable to any AChR antagonists, while the cortex was least vulnerable and only responsive to a higher dose of non-selective nAChR antagonist. Carbachol showed significantly less accumulation of microglia in the hippocampus than the saline controls ($p < 0.01$) in hypoxia–ischemia. *Conclusion:* An AchR-responsive pathway in the brain plays an important role in modifying perinatal brain damage, in which microglial accumulation may be involved.

Keywords: Acetylcholine receptor; Brain damage; Newborn rat; Hypoxia-ischemia; Microglia

1. Introduction

Perinatal hypoxic-ischemic brain damage still remains one of the most important medical problems. The mechanisms involved to develop brain damage have been extensively studied and inflammatory response is one contributor to hypoxic-ischemic brain damage [1]. Recently, we have also demonstrated that peripheral injection of carbachol, known as an acetylcholine receptor (AChR) agonist, reduces brain damage in a newborn rat model of hypoxia-ischemia [2]. However, the role of AChR in the developing brain against hypoxia-ischemia is currently inconclusive. For example, whether AChR antagonists have enhancing effects on hypoxic-ischemic brain damage has not been throughly studied. Besides, since carbachol does not cross the blood–brain-barrier under physiological conditions, it may act through systemic effects, it may stimulate afferent vagus activities to elicit central effects, or hypoxia-ischemia may damage the blood–barain-barrier to permeate it.

In the peripheral organs, macrophage is involved in the regulation of the hypoxia-induced inflammatory pathway via vagus nerve activity [3,4]. Similarly, in the central nervous system, microglia is activated by hypoxia-ischemia [5], which is also related to inflammatory processes by alpha-7-nicotinic-AChR on microglia [6,7]. Thus, we speculated that carbachol, an AChR ago-

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nist, stimulates cholinergic anti-inflammatory pathway via afferent vagus activities, resulting in protection against brain damage in our previous study. However, these interactions between microglial activation and AChR stimulation are currently unclear in the developing brain.

Therefore, we hypothesized that AChR antagonists have deleterious effects on hypoxic-ischemic brain damage in the newborn rat, and that an AChR agonist, carbachol, has protecting effect through modifying microglial activities in the brain after hypoxia-ischemia.

2. Material and methods

Animal model: This study was performed in accordance with the Guidelines of the Experimental Animal Center of the University of Miyazaki, Faculty of Medicine. Pregnant Wistar rats were housed in the same animal center with free access to water and food under a 12-h on/off lighting schedule. The pups were reared with their dams until the time of the experiment.

The 7-day-old rat, whose cerebral maturity corresponds to a 32∼34 week gestation human fetus or newborn infant [8,9], is subjected to Levine-Rice preparation [10,11] to investigate the mechanism of perinatal hypoxic-ischemic brain damage. Briefly, the rats were lightly anesthetized with ether inhalation, and the left common carotid artery was doubly ligated with 5–0 surgical silk. After operation, the pups were allowed 2 h to recover in the incubator without their dams.

In one experiment, pups received a subcutaneous injection of AChR antagonists or saline just before hypoxia to investigate the deleterious effects of AChR antagonists. Our previous study showed that 2-h hypoxia caused brain damage in more than 80% of controls [2]. Thus, pups were subjected to 1-h hypoxia because 2-h hypoxia is too severe to investigate the deleterious effects of AChR antagonists. The AChR antagonists are nonselective nicotinic-AChR antagonist (mecamylamine, (MCA), 5 mg/kg ($n = 19$) and 10 mg/kg ($n = 10$)), selective alpha-7-nicotinic-AChR antagonist (methyllycaconitine, (MLC), 5 mg/kg ($n = 19$) and 10 mg/kg ($n = 11$), and muscarinic receptor antagonist (atropine sulfate, 10 mg/kg, $n = 17$). These doses were determined in reference to doses for adult rats [12–14] and the least dose of the maximum effect and its half dose were chosen for this study. Those AChR antagonists can cross the blood brain barrier [15–17]. We injected AChR antagonists in a water solution. An equivalent volume of saline was given to another 16 pups for saline controls.

In another experiment, the rats received a subcutaneous injection of AChR agonist (carbachol, 0.1 mg/kg, $n = 10$) following our previous study (2) or saline as control ($n = 10$) just before hypoxia to investigate microglia accumulation in the hippocampus. Here, pups were exposed to 2-h hypoxia because 1-h is too mild to see the ameliorating effect of carbachol on brain damage. To compare the degree of acumination of microglia, we also performed immunohistochemical study in rats those were received 2-h hypoxia without ligation ($n = 4$; Hypoxia only) and rats those were received the ligation without hypoxia ($n = 5$; Ligation only).

In both experiments, hypoxia was induced in a chamber perfusing a mixture of humidified 8% oxygen balanced with nitrogen at 33 °C. After hypoxia, pups were returned to their dam.

Rats were sacrificed after 7 days of hypoxia-ischemia by a lethal dose of pentobarbital (100 mg/kg) for histological evaluation. The brains were removed and fixed in a 19:1 solution of ethanol and acetic acid overnight. They were then dehydrated and embedded in paraffin. From each brain, a section 6 μm in thickness was cut that contained the dorsal hippocampus at the A 2.0-mm level in accordance with a stereotactic atlas of a 10-day-old rat brain [18]. Each coronal section was stained with hematoxylin-eosin.

In this model, brain damage is usually confined to the ligated side and the non-ligated side served as control. Once the entire brain in a single section was acquired as an image by the computer, we measured the area of both sides using Image J software. The area of the ligated side was divided by the area of non-ligated side to obtain a ratio as the relative difference of hemisphere area. A value of 100% implies no shrinkage of the ligated side, while 0% implies total loss.

For each hippocampal CA1, CA3, and cortex, we also evaluated regional neuronal damage by microscopic visual observation using a magnification of 150–400 (Fig. 1). The neuronal damage was recognized by selective necrosis (the cytoplasm was stained red and the nucleus was shrunken with less basophilic staining) or infarction (all elements were destroyed with or without cystic formation) using hematoxylin-eosin staining. We identified the damaged area in the ligated side and compared it with the non-ligated side. The severity was expressed using a 3-grade scale following our previous study [19]: mild, <25% of the surface area on a single section with neuronal damage; moderate, 25 ∼ 50%; and severe, >50%.

Microglial accumulation was evaluated 24 h after hypoxia-ischemia since our pilot study showed that microglia was prominent in the hippocampus 24 h after hypoxia-ischemia (unpublished data). A thin paraffin-embedded brain section was washed with water and incubated in 3% hydrogen peroxide for 10 min to inactivate the endogenous peroxidase. Each section was then washed with PBS and blocked with 0.5% bovine serum albumin for 30 min. They were immunohistochemically stained with biotinylated Lycopersicon esculentum tomato lectin (1:100) (Vector Laboratories) for 1 h. Avidin binding was performed using a Vectastain ABC kit and developed using 3,3′-diaminobenzidine as a peroxidase substrate (Vector Laboratories).