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Research Article

Isoflurane anesthesia results in reversible ultrastructure and occludin tight junction protein expression changes in hippocampal blood–brain barrier in aged rats

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HIGHLIGHTS

- Isoflurane anesthesia resulted in reversible hippocampus BBB disruption in aged rats.
- BBB disruption occurred in parallel with down-regulation of occludin expression.
- Isoflurane anesthesia caused cognitive dysfunction.

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ABSTRACT

The underlying mechanism of isoflurane-induced cognitive dysfunction in older individuals is unknown. In this study, the effects of isoflurane exposure on the hippocampal blood–brain barrier (BBB) in aged rats were investigated because it was previously shown that BBB disruption involves in cognitive dysfunction. Twenty-month-old rats randomly received 1.5% isoflurane or vehicle gas as control. Hippocampal BBB ultrastructure was analyzed by transmission electron microscopy and expression of tight junction proteins was measured by western blot analysis. BBB permeability was detected with sodium fluorescein extravasation and further confirmed by immunoglobulin G immunohistochemistry. Spatial learning and memory were assessed by the Morris water maze test. Isoflurane anesthesia resulted in reversible time-dependent BBB ultrastructure morphological damage and significant decreases in expression of the tight junction proteins occludin, which contributed to sodium fluorescein and IgG leakage. Rats with isoflurane exposure also showed significant cognitive deficits in the Morris water maze test. This *in vivo* data indicate that occludin down-regulation may be one of the mediators of isoflurane-induced hippocampus BBB disruption, and may contribute to hippocampus-dependent cognitive impairment after isoflurane exposure in aged rats.

1. Introduction

Postoperative cognitive dysfunction (POCD) is a common complication in older patients undergoing surgery [6]. Recently, inhalational anesthetics such as isoflurane have been shown to induce neurotoxicity and contribute to cognitive impairment [24], but the exact cascade leading to its development is unclear.

The blood-brain barrier (BBB) is formed by highly organized endothelial cells, which precisely regulate the homeostasis of the central nervous system (CNS) [26]. BBB disruption is involved in cognitive impairment in a variety of animal models including Alzheimer's disease (AD) [26], cerebral ischemia [3], hypertension [13] and type 2 diabetes mellitus [1]. BBB disruption has been linked to proinflammatory cytokine transport and β-amyloid (Aβ) clearance [26]. Thus, BBB disruption may play a critical role in the progression of cognitive impairment. Furthermore, previous studies from our laboratory [10,12] and others [5] have suggested that

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inhalational anesthetics may alter cognitive function *via* increased neuroinflammation, Aβ accumulation, and dysregulated mitochondrial homeostasis with subsequent cellular apoptosis. Therefore, from a neurocognitive standpoint, we speculated that isoflurane may attribute to neurological disorders through the disruption of BBB integrity. Moreover, no study has examined in detail the effects of isoflurane exposure on BBB functional and ultrastructural changes in the aged brain.

In the present study, we investigated the effects of isoflurane on hippocampal BBB ultrastructure in aged rats. Previous work reported that tight junction proteins (TJPs) exist in cerebral vascular endothelial cells, and play a critical role in maintaining BBB integrity [15]. We therefore observed the expression of TJPs in response to isoflurane exposure. Furthermore, the effects of isoflurane on BBB permeability and cognitive function were observed.

2. Materials and methods

2.1. Animals

Aged male Sprague-Dawley rats (20 months of age, weighing 500–600 g) were used for the experiments. Rats were bred and maintained under standardized housing conditions with food and water *ad libitum*. The experimental protocol was approved by the Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch (Approval No. LA 2012–38).

2.2. Experiment protocols

To study the effects of isoflurane exposure on BBB disruption, rats were randomly assigned to control ($n = 4$, each) or isoflurane groups (total: $n = 120$) and exposed to vehicle gas or isoflurane, respectively. BBB ultrastructural changes in the CA1 region of the rat hippocampus were examined by a transmission electron microscope (TEM) at 30 min, 1, 2 and 4 h during isoflurane exposure, and 24, 48 and 72 h after isoflurane exposure. Sodium fluorescein (NaFL) uptake and leakage of immunoglobulin G (IgG) in the hippocampus were examined at the same time, using fluorimetry and immunohistochemical semiquantitative analysis, respectively ($n = 8$ per time point). Hippocampus was harvested immediately and 72 h after the 4 h isoflurane exposure, and Western blotting analysis was performed for TJPs expression ($n = 8$ per time point). Twenty-four hours after isoflurane exposure, hippocampus-dependent spatial learning and memory using the Morris water maze (MWM) test ($n = 12$ per group).

2.3. Isoflurane exposure

The protocol for isoflurane exposure was based on our previous studies [10]. Briefly, aged rats were randomized by weight into experimental group that received 1.5% isoflurane (Baxter Healthcare, Deerfield, IL, USA) plus 100% oxygen for 4 h, or a control group that received 100% oxygen. The concentrations of isoflurane, oxygen, and carbon dioxide in the chamber were continuously analyzed with a gas monitor (Datex–Ohmeda, Louisville, CO, USA). This anesthesia protocol does not cause significant changes in blood gas and blood glucose [10].

2.4. Ultrastructural analysis

As described previously [8], the sections of hippocampal CA1 region were washed with cacodylate buffer and immersed in osmium tetroxide solution, and stained with uranyl acetate and lead citrate. TEM (JEM-1400, Electron Co., Japan) was used to observe the ultrastructure changes in the basal laminas, tight junctions (TJs), mitochondria, the endoplasmic reticulum (ER) and the angioedema surrounding the capillaries that are indicative of BBB integrity disruption. Assessments were made by an independent observer blinded to the study.

2.5. Western blot analysis

TJPs of hippocampal BBB markers expression were examined by western blots using the following primary antibodies: anti-claudin-5 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-occludin (1:500; Santa Cruz Biotechnology) and anti-ZO-1 (1:500; Santa Cruz Biotechnology). Fluorescently labeled secondary antibodies (1:10,000; LI-COR Biosciences, Lincoln, NE, USA) were used.

2.6. Measurement of NaFL extravasation

NaFL (MW ≈ 376.27 Da) assay was carried out as previously described [7]. 10% NaFL (0.6 ml/kg, Sigma, St. Louis, MO, USA) was injected *via* the tail vein and allowed to circulate for 30 min. Then the hippocampus were harvested and homogenized in 3 ml of cold 7.5% trichloroacetic acid (Sigma) and centrifuged at 10,000 × g for 10 min. NaFL (excitation, 485 nm; emission, 535 nm) concentrations in supernatants were analyzed using a fluorescent microplate reader. The results were expressed as μg/mg of brain tissue against standard curves.

2.7. Immunohistochemical staining for IgG protein expression

IgG (MW ≈ 150 kDa) immunohistochemical staining was performed as previously described [20]. Brain sections (20 μm) were incubated with rabbit anti-rat IgG (1:800; Santa Cruz Biotechnology) primary antibody. Horseradish peroxidase-labeled goat anti-rabbit (1:400; Santa Cruz Biotechnology) secondary antibody was used. Semi-quantitative analysis was performed at 200× magnification per visual field (0.145 mm^2) for IgG extravasation, using imaging software (ImagePro Plus 6.0; Media Cybernetics, Bethesda, MD, USA). The mean IOD values were analyzed and averaged.

2.8. MWM test

Twenty-four hours after isoflurane exposure, spatial learning and memory was evaluated by the MWM test (Sunny Instruments Co. Ltd., Beijing, China) as previously described [9,10]. Briefly, the rats received four training trials daily for 5 consecutive days. The time to locate the submerged platform and swimming speed were measured. On test day 6, a probe trial was conducted in which rats were tested in the absence of the platform. Time spent in the target quadrant and platform site crossovers was analyzed.

2.9. Statistical analysis

For statistical analyses, SPSS 16.0 for Windows (SPSS, Chicago, IL) was used. Data collected from the behavioral studies were analyzed using two-way repeated-measures ANOVA, with Bonferroni post hoc analysis. All other data were analyzed with a one-way ANOVA comparing each time point to the control. Data were expressed as mean ± SEM and statistical significance was set at $p < 0.05$.

3. Results

3.1. Isoflurane exposure altered the morphology of BBB ultrastructure

In the control group, after 30 min and 1 h of isoflurane exposure, the ultrastructure of the basal laminas was continuous and