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Endoplasmic reticulum stress pathway mediates isoflurane-induced neuroapoptosis and cognitive impairments in aged rats

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HIGHLIGHTS

- Isoflurane induces hippocampal neuroapoptosis and cognitive impairments in aged rats.
- Isoflurane triggers ERS in the hippocampus of old rats.
- ERS-mediated apoptosis pathway is involved in isoflurane-induced neurotoxicity.
- Attenuation of ERS protects against isoflurane-induced neurotoxicity.

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ABSTRACT

Postoperative cognitive dysfunction (POCD) is increasingly being recognized as an important clinical syndrome. Although it has been documented that volatile anesthetics induce neuronal apoptosis and cognitive deficits in several aged animal models, the underlying mechanisms are not well understood. Endoplasmic reticulum stress (ERS) is considered as an initial or early response of cells under stress and linked to neuronal death in various neurodegenerative diseases. The study was designed to explore the possible role of ERS pathway in isoflurane-induced neuroapoptosis and cognitive impairments. In the present study, twenty-month-old rats were exposed to 1.3% isoflurane for 4 h. Two weeks later, the rats were subjected to behavioral study. Protein and mRNA expressions of ERS markers were evaluated. Meanwhile, hippocampal neuronal apoptosis was also detected. We found that isoflurane triggered ERS as evidenced by increased phosphorylation of eukaryotic initiation factor (EIF) 2α, and increased expression of 78-kDa glucose-regulated protein (GRP78), activating transcription factor (ATF) 4 and C/EBP homologous protein (CHOP). Furthermore, the level of apoptosis in the hippocampus was significantly up-regulated after isoflurane exposure, and salubrinal (ERS inhibitor) treatment attenuated the increase. More importantly, cognitive impairments caused by isoflurane were also effectively alleviated by salubrinal pretreatment. These results indicate that ERS-mediated apoptotic pathway is involved in isoflurane neurotoxicity in aged rats. Inhibition of ERS overactivation contributes to the relief of isoflurane-induced neurohistopathologic changes.

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1. Introduction

Postoperative cognitive dysfunction (POCD) is increasingly being recognized as an important clinical complication of major surgery, which predisposes to impairments in patients' daily functioning and an increased perioperative morbidity and mortality [1]. Technological development in surgery and anesthesia has achieved great advances during the last few decades, however, the incidence of POCD stills relatively high, especially in the elderly [2,3]. Although POCD is likely to be transient or self-limiting in most patients, in some patients, it can be long-term or even permanent [3,4]. POCD has drawn significant attention from the public and scientific community.

In today's medical practice, in pursuit of comfort and safety, most patients choose to have surgery under general anesthesia, especially geriatric surgical patients [5]. Inhalation anesthetics such as isoflurane have been widely used in recent years in clinical and research practices. There is no clear clinical evidence to indicate the link between anesthesia and cognitive deficits, however, a growing body of preclinical evidence supports the view that exposure to general anesthetics may contribute to POCD [6–8]. The phenomenon is receiving increasing attention. However, the underlying pathophysiological mechanisms of isoflurane-induced hippocampal neuronal loss and cognitive impairments are

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complex and still not completely established. It is acknowledged that ERS is acted as an early or initial response of cells to stress or damage and related to neuronal injury or death in several neurodegenerative diseases [9,10]. Moderate ERS serves as a protective mechanism through activating unfolded protein response (UPR), but prolonged or severe stress can trigger apoptotic pathways [11–13]. C/EBP homologous protein (CHOP), a key proapoptotic transcription factor, plays a critical role in ERS-induced apoptosis [12].

In the current study, using an old rat model, we explored the role of ERS pathway in isoflurane-induced hippocampal neuroapoptosis. Salubrinal, a selective inhibitor of eukaryotic translation initiation factor 2 subunit α (eIF2α) dephosphorylation, is known for its ability to protect cells from ERS-induced apoptosis [14], and pretreated by intraperitoneal injection to examine the relationship between ERS pathway and isoflurane-induced neuronal apoptosis, even cognitive impairments. Furthermore, the possible signaling pathways of ERS-associated apoptosis were assessed to clarify the mechanism of isoflurane-induced hippocampal apoptosis and cognitive impairments.

2. Materials and methods

2.1. Animals

All animals were treated in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals (United States National Institutes of Health). All animal experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of Zhejiang University (Zhejiang, China). All efforts were made to minimize the number of animals used and their suffering. Twenty-month old male clean healthy Sprague–Dawley rats, weighing 350–400 g, were used for all experiments. The animals were housed in polypropylene cages with food and water available ad libitum, and the room temperature was maintained at 22 °C, with a 12-h light–dark cycle.

2.2. Isoflurane exposure

The rats were randomly divided into four groups: control group (CON), isoflurane group (ISO), isoflurane plus salubrinal group (ISO + SAL) and salubrinal group (SAL) (n = 20 in each group). Salubrinal was dissolved in DMSO and intraperitoneally injected 30 min before exposure to isoflurane (1 mg/kg, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Rats in the other two groups were intraperitoneally injected with an equal volume of DMSO. To induce general anesthesia, rats were placed in a translucent plastic chamber (length, 30 cm; width, 43 cm; and height, 14 cm) within a thermostatic bath (38 °C). Each plastic container has two interfaces, one is connected to anesthesia machine and the other is connected to a multi-gas monitor. For animals in the isoflurane and isoflurane plus salubrinal group, 1.3% isoflurane was delivered by a humidified 30% O_2 carrier gas from a calibrated vaporizer. Control experiments were performed in the same treatment and environment, except no isoflurane was added when flushing the chamber. The carrier gas was delivered at a flow rate of 2 l/min. The concentrations of isoflurane, O_2 and CO_2 in the chamber were monitored continuously with a calibrated Datex Capnomac Ultima Gas Analyzer (Datex Ohmeda, Helsinki, Finland). The rectal temperature was maintained at 37 ± 0.5 °C. Mean blood pressure (MAP) and heart rate of animals were measured with a CODA Monitor (Kent Scientific Corp., Torrington, CT, USA). CODA Monitor is a non-invasive method to measure the blood pressure in rats by determining the tail blood volume using a volume pressure recording sensor and an occlusion tail-cuff, which is largely unaffected by slight movement of animals and has equal accuracy, consistency, and reliability in awake or anesthetized animals. The MAP and heart rate were recorded every hour. Arterial blood gases (ABG) and blood glucose were measured at the end of the 4-hour anesthetic exposure.

At 4 h after exposure, 6 rats from each group were euthanized by overdose Nembutal and the left hippocampus was dissected for neuronal apoptosis examination and the right was examined for protein or mRNA analysis. And the rest of animals were subjected to behavior study to determine their cognitive function after two weeks.

2.3. Western blot analysis

Brain tissues were harvested and were homogenized on ice with 2 mM phenylmethanesulfonyl fluoride in 1 ml ice-cold RIPA buffer added protease inhibitor cocktail EDTA-free. Homogenates were centrifuged at 13,000 ×*g* at 4 °C for 30 min. The supernatant was saved and its protein concentration was determined by the BCA method using bovine serum albumin as the standard. Protein samples (50 μg) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Wuhan Boster Biological Technology, Ltd, China). The membranes were blocked by nonfat dry milk buffer for 2 h and then incubated overnight at 4 °C with primary antibodies anti-caspase-3 (1:1000; Cell Signaling Technology, Beverly, MA, USA), Bax (1:1000; Abcam, Cambrige, UK), Bcl-2 (1:500; Abcam, Cambrige, UK), GRP78 (1:1000; Santa Cruz, CA, USA), total EIF2α (1:1000; Santa Cruz, CA, USA), ATF4 (1:1000; Santa Cruz, CA, USA), phospho-EIF2α (1:1000; Santa Cruz, CA, USA), and CHOP (1:1000; Cell Signaling Technology, Beverly, MA, USA). The membranes were subsequently incubated with HRP-conjugated secondary antibodies and detected with enhanced chemiluminescence (ECL) detection reagent (Amersham Biosciences, Piscataway, NJ). The bands were digitally scanned and analyzed using ImageJ software (NIH image, National Institutes of Health, Bethesda, MD). The results were expressed as a relative density. Equal protein loading in each lane was confirmed by hybridization with a 1:10,000 dilution of β-actin antibody (Cell Signaling Technology, Beverly, MA, USA).

2.4. Quantitative RT-PCR analysis

Total RNA was extracted from hippocampus with TRIzol Reagent.

cDNA was synthesized from 1 μg of RNA using the PrimeScript RT reagent Kit (Takara Biotechnology, Dalian, China) following the instructions provided by the manufacturer. Amplification was carried out on 7500 quantitative PCR System (Applied Biosystems) using SYBR Premix Ex Taq (Takara Biotechnology) according to the manufacturer's instructions. The primers used for quantitative PCR were as follows: GRP78 (forward: ACGACCGCTAACGAAGTCAC 5′–3′, reverse: ATCATGACCACA GTCCATGC 5′–3′); ATF4 (forward: AGA ATG GCA GCA CAG ACT GCG CG 5′–3′, reverse: GGA ACT GGT CGA TCT GGG TA 5′–3′); CHOP (forward: GTACGACCGCTAACGAAGTC 5′–3′, reverse: ATCTCCATGGTGGT GCATCAT 5′–3′); GAPDH (forward: GAACGACCGCTAACGAAGTC 5′–3′, reverse: GGTGAATCATGACCACAGTCC 5′–3′). The fold change in expression of each gene was calculated using the ΔΔCt method, with the housekeeping gene GAPDH mRNA as an internal control.

2.5. Nissl staining

The hippocampus was fixed in 10% neutral buffered formalin overnight and then paraffin embedded. Coronal 8 μm sections were prepared and subjected to Nissl staining. These sections were examined by an observer blinded to the group assignment of the sections. Three sections from each animal were selected at random and the number of positive cells (neurons) in the CA1 regions under a high magnification field (×400) in 5 visual fields/per section was counted. The densities of neurons were measured quantitatively using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

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