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Research report

Role of autophagy in sevoflurane-induced neurotoxicity in neonatal rat hippocampal cells

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ABSTRACT

Background: Sevoflurane has been extensively employed for induction and maintenance of general anesthesia. The *effect* of sevoflurane-induced apoptosis in developmental neurotoxicity has been appreciated for some time now, but the underlying *mechanism* of developmental neurotoxicity has not been established. The aim of our study is to evaluate the role of autophagy in sevoflurane-induced neurotoxicity through observing changes in the levels of autophagy in hippocampal neurons after exposure to sevoflurane.

Methods/Materials: Primary cultured hippocampus neuronal cells were exposed to either 3.4% sevoflurane for 1 h (S_{1h} group), 3 h (S_{3h} group), 5 h (S_{5h} group), or air (control group). We observed changes in autophagy proteins Beclin-1, LC3-II, p62, and Beclin-1mRNA, LC3mRNA and SQSTM1mRNA using Western Blot and QRT-PCR. We also determined the expression of LC3 using immunofluorescence staining, monitored the occurrence of autophagy using RFP-GFP-LC3 expression plasmid transient transfected hippocampal neuronal cells, detected the expression of LC3-II using siRNA Knockdown Beclin-1 and Atg5, and determined changes in cell apoptosis using Annexin V/PI staining and flow cytometry.

Results: After primary cultured hippocampal neuronal cells were exposed to 3.4% sevoflurane for 5 h, the expression level of Beclin-1 and LC3-II increased and p62 decreased in Western blotting. The expression of Beclin-1mRNA, LC3mRNA increased and SQSTM1mRNA decreased in QRT-PCR. LC3 increased with cell immunofluorescence staining, LC3 expression plasmid increased after mRFP-GFP-LC3 expression plasmid transient transfection and LC3-II decreased after transfection with siRNA Beclin-1 and siRNA Atg5. The apoptosis rate of primary cultured hippocampal neuronal cells increased in Annexin V/PI staining and flow cytometry analysis.

Conclusion: This study demonstrates that sevoflurane may induce hippocampal neuron autophagy in primary cultured hippocampal neuronal cell and that Beclin-1 and Atg5 are involved in the process of sevoflurane-induced autophagy. Exposure of sevoflurane may not only induce autophagy of hippocampal neurons but also activate the apoptosis of hippocampal neurons. Autophagy may play an important role in sevoflurane-induced neurotoxicity in primary cultured hippocampal neuronal cells.

Clinical implications

What is already known

- Early exposure to commonly used anesthetic agents such as sevoflurane can lead to widespread hippocampal neuronal apoptosis and persistent spatial learning deficits in the developing rat brain.

What this study adds

- Sevoflurane may induce hippocampal neuronal autophagy in primary cultured hippocampal neuron cells. Beclin-1 and Atg5 is involved in the process of sevoflurane-induced autophagy. The exposure of hippocampal neuronal cells to sevoflurane may not only induce autophagy but also activate apoptosis of these neurons. Autophagy may play an important role in sevoflurane-induced

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neurotoxicity in primary cultured hippocampal neuronal cells.

1. Introduction

Procedures related to advances in pediatric neurology and neurosurgery require infants and children be exposed, sometimes frequently, to a variety of general anesthetic agents (GAs), such as sevoflurane, that have been demonstrated to modulate neuronal activity in key stages of brain development. Clinical studies (Yang et al., 2012) demonstrate that sevoflurane can cause significant long-term behavioral deficits and cognitive impairment in children. Animal studies (Wang et al., 2013) have shown that early exposure to sevoflurane can lead to widespread hippocampal neuroapoptosis and persistent spatial learning disabilities in developing rats. Current evidence (Joseph et al., 2014) indicates that inhalational anesthetics induce cell damage by causing abnormal calcium release from the endoplasmic reticulum (ER) via over-activation of inositol trisphosphate (InsP3Rs) and/or ryanodine receptors (RyRs) in a concentration- and duration-dependent manner. The specific mechanism of sevoflurane-induced developmental neurotoxicity, however, is still not clear. To devise preventative and protective strategies, these mechanisms must be identified.

Autophagy is a natural degradation process which plays an important role in the metabolism of cellular organelles and proteins (Jevtovic-Todorovic et al., 2012). It is the primary means of stabilizing the internal cellular environment by scavenging misfolded proteins and damaged organelles (Jevtovic-Todorovic et al., 2012). GAs induce direct lysosomal functional disturbances and elevate calcium levels which results in endosomal-lysosomal trafficking over activation which gives rise to lipid peroxidation, production of reactive oxygen species (ROS), receptor up-regulation, and mitochondrial damage (Jevtovic-Todorovic et al., 2012). Anesthesia-induced autophagy is being gradually accepted as a protective mechanism focusing on eliminating injured mitochondria. It might also be the result of intracellular calcium signaling derangements due to endoplasmic reticulum (ER) dysfunction (Xu et al., 2018). Sanchez et al. demonstrated that general anesthesia can cause long-term impairment of both mitochondrial morphogenesis and synaptic transmission in the developing rat brain, both of which are accompanied by enhanced autophagy activity (Sanchez et al., 2011). It is speculated that autophagy may play an important role in sevoflurane neurotoxicity. The aim of our study is to investigate changes in the levels of autophagy in neonatal hippocampus neuronal cells induced by sevoflurane and to pinpoint the role of autophagy in sevoflurane-induced neurotoxicity. We also aimed to identify related mechanisms as well as any relationship between autophagy and apoptosis of neurons to identify therapeutic targets and determine the safest clinical exposure time.

2. Materials and methods

2.1. Materials

Polyclonal rabbit antibodies including anti-LC3, anti-Beclin-1, anti-P62, anti-Bcl-2, and anti-Bax were obtained from Cell Signaling Technology (MA, USA).

Neurobasal medium, HBSS, B27 serum-free supplement, Penicillin-Streptomycin, Glumax, Trypsin-EDTA, and horse serum were all obtained from GIBCO, Invitrogen GmbH (Karlsruhe, Germany).

2.2. Primary neuronal culture

Cultures of primary hippocampal neurons were performed as described (Zhao et al., 2017). Postnatal day 1 Sprague-Dawley rats were obtained from Zhejiang Chinese Medical University (2015000511349) and used for cell culture preparation. Laboratory Animal Care and Use Guidance Suggestions determined by The China Science and Technology Ministry guided all experimental procedures (The Ministry of Science and Technology, 2006). After removing meninges, cerebral hippocampi were separated from brains of 12 rats in each group and dissected in HBS. Tissue fragments were added to a dissociation medium (Neurobasal medium 48 ml with 2% B27 supplement 1 ml, 1% horse serum 0.5 ml, and 0.25% Glumax 0.5 ml) and subjected to mechanical dissociation by repeated aspirations through a Pasteur pipette after mild trypsinization 20 min in 0.25% Trypsin-EDTA at 37 °C. The cell suspension was pelleted by mild centrifugation (2000 rpm for 3 min at room temperature) followed by seeding in 3.5 cm culture dishes in the dissociation medium at a specific gravity of 3×10^5 cells per ml. Before cell plating, every cell culture dish was pretreated with 0.1% poly-D-lysine (Sigma, St Louis, Missouri, USA) at room temperature for 2 h followed by rinsing twice with phosphate-buffered saline (PBS). Cells were kept in an incubator under 5% CO_2 at 37 °C. The medium was replaced with serum-free medium (Neurobasal medium 48 ml with 2% B27 supplement 1 ml, Penicillin-Streptomycin 0.5 ml, and 0.25% Glumax 0.5 ml) within 24 h. With indirect anti-NeuN immunocytochemistry assay, each cell culture dish demonstrated 99% neuronal cells (Smothers et al., 2016).

2.3. Anesthetic agent treatment

After 7 days, primary cultured hippocampus neurons cells in culture dishes were allocated by computer-generated random numbers into 4 groups (n = 12 each). Each group of dishes were placed in an anesthesia induction chamber maintained at 37 °C (RWD Life Science Co., Ltd., Shenzhen, China) containing fresh gas (21% O_2, 5% CO_2 and 69% N_2) (group C) or with the addition of 3.4% sevoflurane for 1 h (group S1 h), 3 h (group S3 h), and 5 h (group S5 h). A steady level of 3.4% sevoflurane was maintained with a gas flow rate of 1 l/min and measured by a Capnomac gas monitor (Datex-Ohmeda, Helsinki, Finland).

2.4. Western Blot analysis for Beclin-1, LC3B, p62, Bcl-2, and Bax

After treatment with sevoflurane, cells were collected and washed twice with cold PBS and lysed on ice in RIPA lysis buffer (Boster Biotechnology, Wuhan, China) containing phenylmethanesulfonyl fluoride (Boster Biotechnology, Wuhan, China) for 15 min. The cell then underwent ultrasonic shattering (Vibra cell, Sonic, USA). The supernatant was collected after centrifugation (13,200 rpm for 15 min at 4 °C), and the concentration of protein was measured with a BCA kit (Beyotime Biotechnology, Haimen, China). Samples (40 μg protein) were prepared using primary cultured hippocampus neuronal cells. To these was added sample buffer, then separated with 12% sodium dodecyl sulfate-polyacrylamide gel, and electroblotted to a nitrocellulose membrane (Bio-Rad, USA). The membrane was blocked with blocking solution (5% nonfat milk in Tris-buffered saline with Tween 20 [TBST]) at room temperature for 1 h. Blots were incubated overnight at 4 °C with specific rat monoclonal antibodies: anti-LC3 B (2775), anti-Beclin-1 (3495), anti-P62 (5114), anti-Bcl-2 (2870), anti-Bax (14796), and anti-GAPDH (5174) (1:1000 dilution; Cell Signaling Technology, Beverly, MA, USA). After being washed three times, samples were incubated with a horseradish peroxidase-labeled second antibody goat anti-rat antibody (1054) (1:5000 dilution; Boster Biotechnology, Wuhan, China) for 2 h at room temperature followed by visualization with chemiluminescence detection (SuperSignal West Pico Chemiluminescent Substrates; Pierce Biotechnology, Rockford, IL, USA). Protein band absorbance (GEL-PRO ANALYZER software; Bio-Rad Laboratories, Hercules, CA, USA) was quantified with densitometric techniques and expressed as relative densitometric units compared to the matching control.

2.5. Quantitative real-time polymerase chain reaction (QRT-PCR) assay for Becn 1, LC3, and SQSTM1

Total RNA was extracted with trizol reagent RNAiso Plus (Takara